

THE IMPACT OF DIET-INDUCED OBESITY AND EXERCISE ON
ADIPOSE-RESIDENT STEM CELLS

BY

YAIR PINCU

DISSERTATION

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Doctoral Committee:

Associate Professor Marni Boppart, Chair
Professor Jeffrey Woods
Associate Professor Kenneth Wilund
Professor Timothy Koh, UIC

ABSTRACT

Recent studies suggest that vascular-associated multipotent stem/stromal cells exist in adipose tissue that can influence extracellular matrix (ECM) remodeling during lipid filling and adipose expansion. Both mesenchymal stem cells (referred to as adipose-derived stromal cells, or ADSCs) and pericytes may secrete factors such as collagen and metalloproteinases that can allow for tissue turnover. Therefore, the primary goal of this dissertation was to examine the impact of long-term high fat feeding on vascular-associated stem/stromal cell quantity and function, as well as the capacity for endurance exercise to mitigate these responses. Two studies were conducted. In the first, male C57BL/6J mice were placed on control or high fat diet (HFD) for 8 wk prior to and following initiation of a 16 wk treadmill exercise program. ADSCs (Sca-1⁺CD45⁻) were isolated from epididymal adipose tissue and mRNA was evaluated using high throughput qPCR. While resulting in a total increase in ADSC quantity, HFD decreased the relative quantity of ADSCs and markedly altered gene expression related to ECM remodeling (Col1 α 1, MMP2, MMP9, Timp1). Exercise did not reverse these changes. In the second study, a multiplex flow cytometry strategy was developed to evaluate the pericyte fraction using the prototypical proteoglycan cell surface marker, neural/glial antigen 2 (NG2), including two pericyte subtypes based on Nestin expression (+/-). Male and female NG2-DsRed x Nestin-GFP reporter mice were placed on control or HFD for 6 wk prior to and following initiation of a 12 wk treadmill exercise program. NG2⁺Nestin⁻ (Type 1 pericyte) and NG2⁺Nestin⁺ (Type 2 pericyte) cells were isolated from inguinal adipose tissue of these double transgenic animals, and quantity was evaluated after gating for endothelial (CD31⁺) and hematopoietic cells (CD45⁺) (Lin fraction) using flow cytometry. Delineation of subtype quantitation was not pursued due to the fact that Type 2 pericyte quantity was very low in adipose tissue. Total NG2⁺Lin⁻ pericyte

quantity was increased in males, but not in females, in response to HFD. Interestingly, exercise suppressed the rise in pericyte quantity in males. HFD did not significantly alter cell surface marker expression, and adipogenic progenitor cell marker expression remained low.

In conclusion, the results of these studies suggest that ADSC relative quantity is decreased and adipose-resident NG2⁺ pericyte relative quantity is increased in response to long-term HFD. Based on gene expression and cell surface marker expression data, engagement in adipogenesis does not likely account for the alteration in adipose-resident stem cell quantity due to the obese condition. Striking changes in ECM gene expression suggest that ADSCs strongly contribute to ECM remodeling during tissue expansion. Finally, the fact that ECM gene expression was not mitigated by exercise training may imply that full recovery of adipose tissue health is dependent on a restriction in energy intake.

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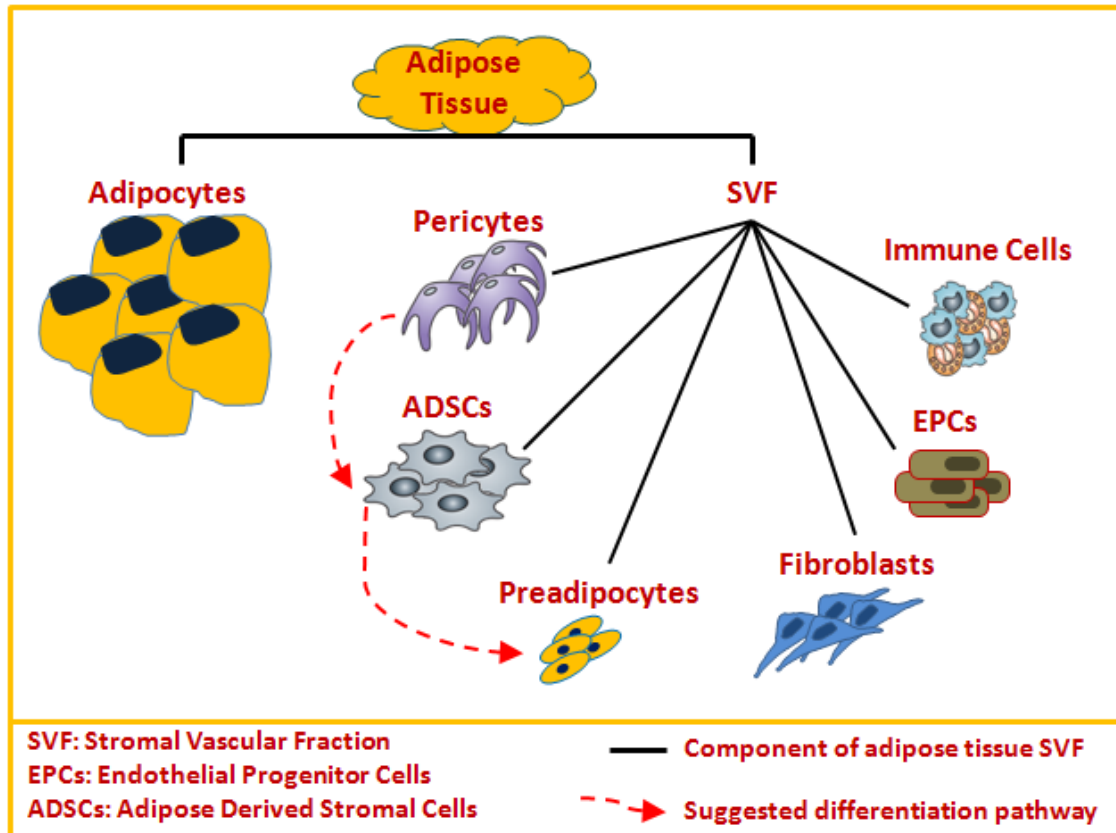
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CHAPTER 1: INTRODUCTION

Obesity is a rapidly increasing epidemic with over one third (36%) of American adults reporting a BMI of 30 or higher [1]. Obesity is associated with increased systemic inflammation that results in metabolic dysfunction, including Type 2 Diabetes. Obese individuals have increased risk of all-cause and cardiovascular disease mortality [2]. It is clear that obesity presents a major public health challenge, evident by the immense medical care cost of obesity in the U.S., reported at \$147 billion in 2008. In a recent publication, an astonishing prediction was made that 51% of the world population will be obese by 2030, resulting in a \$549.5 billion increase in medical expenses in the next two decades [3]. Increased nutrient consumption results in adipose tissue expansion through adipocyte hypertrophy, hyperplasia, or both [4-6]. Adipose tissue expansion involves carefully orchestrated extracellular matrix (ECM) remodeling, angiogenesis and adipogenesis [6-9]. These processes become dysfunctional in the obese condition, resulting in adipose tissue dysfunction, lipotoxicity and systemic inflammation, which are likely to cause metabolic impairments, including type 2 diabetes [4-5, 8]. In addition to adipocytes, the stromal vascular fraction (SVF) of adipose tissue includes immune cells, fibroblasts, preadipocytes, and important vascular-associated stem/stromal cells (see Figure 1.1), including adipose-derived stromal cells (ADSCs), predominantly mesenchymal stem cells (MSCs) (CD45⁻CD31⁻CD146⁻NG2⁻) and pericytes (CD45⁻CD31⁻CD34⁻CD146⁺NG2⁺) [7-8, 10]. These different cell populations must work in concert to maintain adipose tissue health and to remodel adipose tissue in order to maintain or to increase lipid storage capacity [7-8]. The complex cell-cell interactions that occur within the SVF to allow for appropriate tissue remodeling and expansion during conditions of healthy weight gain or obesity are not fully understood.

Figure 1.1 Cellular Components of Adipose SVF



Sca-1 (Stem cell antigen-1) is a glycosyl phosphatidylinositol-anchored cell surface protein expressed by stem/stromal cells in a variety of tissues, including mesenchymal stem cells (MSCs) and pericytes [11-15]. Recent studies suggest that Sca-1⁺Lin⁻ (Lin: CD45⁻CD31⁻Ter119⁻) cells provide an essential role in adipose tissue remodeling, an event that may be impaired with long-term high fat feeding [16-19].

Pericytes envelop capillaries and microvessels in various organs, including skeletal muscle and adipose tissue [20]. Pericytes are important regulators of vascular function and angiogenesis, yet current studies suggest that pericytes are multipotent stem cells with capacity for differentiation into osteocytes, myocytes and adipocytes [21]. In addition, resident pericytes secrete a wide variety of paracrine factors that may be important for adipose tissue ECM remodeling [21-22]. Studies exploiting different methodologies, including fluorescent activated

cell sorting (FACS), provide evidence that pericytes reside in the microvascular niche of adipose tissue [6, 20, 23-25]. However, a precise role for adipose-resident pericytes in the maintenance of tissue health during excess nutrient intake is unclear. In addition, the extent to which exercise training can alter adipose tissue resident stem/stromal cell (ADSCs/MSCs or pericytes) quantity, phenotype (surface marker expression) or function (paracrine factor synthesis and release) during the condition of long-term high fat feeding is not currently known.

The overall goal of this study is to examine the impact of obesity and endurance training on adipose tissue resident stem/stromal quantity and function. A brief description of the rationale, hypotheses and methods is presented below while a complete account of the methodology can be found in the respective chapters under *Materials and Methods*.

1.1 Specific aims

This study was designed to address the following three aims:

- 1) To evaluate the impact of prolonged high fat diet (HFD), exercise, and their combination on epididymal ADSC (Sca-1⁺CD45⁻) quantity and gene expression (see Chapter 3).
- 2) To determine the relative proportion of Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes in subcutaneous adipose tissue and subsequently characterize common pericyte- and lineage-surface marker expression in the two types (see Chapter 4).
- 3) To evaluate the impact of HFD, exercise, and their combination on subcutaneous adipose-resident NG2⁺ pericyte quantity and cell surface marker expression (see Chapter 4).

Specific Aim 1: To evaluate the impact of prolonged high fat diet (HFD), exercise, and their combination on epididymal ADSC (Sca-1⁺CD45⁻) quantity and gene expression.

Sca-1⁺CD45⁻ adipose-derived stromal cells (ADSCs), a heterogeneous population of cells which include MSCs and pericytes, possess capacity for adipogenesis and release of factor that can dictate tissue health. Studies are necessary to determine the extent to which ADSCs are influenced by lifelong obesogenic behavior and contribute to overall tissue health. Exercise training promotes many positive outcomes such as suppression of adipose tissue inflammation [26-28], macrophage polarization towards M2 phenotype [28], improvements in cellular oxidative homeostasis [27] and most importantly, a reduction in adiposity [26]. The extent to which exercise training can impact ADSCs in the context of obesity is not clear.

In this study, 3 month old, male mice will be subjected to a long duration high fat diet (60% of the calories from fat for 24 weeks) with or without the addition of progressive aerobic exercise (motorized treadmill, 10-17 m/min, 5 d/wk, 60 min/d). The age of the mice and the duration of the intervention mimic a lifelong of obesogenic behavior (HFD with no exercise) or a healthier lifestyle (HFD with exercise or control diet with or without exercise).

At the end of the study, stromal-vascular (SVF) cells will be harvested from adipose tissue and mononuclear cells will be harvested from skeletal muscle and cell suspensions will be enriched for the Sca-1⁺CD45⁻ population using FACS. Sorted cells will then immediately be lysed, RNA will be extracted, and gene expression will be analyzed using qPCR. In addition, whole adipose tissue will be examined for changes in gene expression using qPCR and collagen accumulation using histology.

We hypothesize that HFD will result in adipose tissue inflammation and fibrosis, and that these changes will coincide with decreased ADSC quantity. The decrease in quantity may be due

to increased adipogenic lineage commitment. In addition, HFD will significantly alter ADSC ECM gene expression to reflect engagement in tissue remodeling.

Alternative hypothesis: HFD may stimulate ADSC proliferation. In this case, we would expect to observe a net increase in ADSC quantity. No changes in adipogenic and ECM- related gene expression will suggest that ADSC do not directly contribute to adipogenesis and ECM remodeling.

Specific Aim 2: To determine the relative proportion of Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes in subcutaneous adipose tissue and subsequently characterize common pericyte- and lineage-surface marker expression in the two types

Two pericyte subpopulations were recently identified in skeletal muscle [29-30], one which provides a stromal role in tissue health (Type 1) and one which contributes to tissue repair (Type 2). The results from these studies suggest that Type 1 pericytes in adipose tissue may provide the underlying basis for adipose tissue health in the context of obesity. We recently established a colony of double transgenic reporter mice (Nestin-GFP x NG2-DsRed) which allows for the identification of Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes.

In this experiment, Type 1 and Type 2 pericytes will be extracted from subcutaneous adipose tissue from 8 week old, female double transgenic mice and characterized for common pericyte markers by multiplex flow cytometry. Skeletal muscle will be used as a positive control to verify our findings since the two types of pericytes have been previously established to reside in skeletal muscle. We hypothesize that subcutaneous fat will harbor both pericyte types, and that pericyte surface marker expression will reflect subtypes in skeletal muscle.

Alternative hypothesis: Type 1 and Type 2 pericytes were first characterized in skeletal muscle [29-31] and only Type 1 pericytes were found to possess adipogenic capacity (Type 2 pericytes are myogenic). Thus, it is possible that only the Type 1 (adipogenic) subpopulation will be localized in adipose tissue.

Specific Aim 3: To evaluate the impact of HFD, exercise, and their combination on subcutaneous adipose-resident NG2⁺ pericyte quantity and cell surface marker expression.

HFD results in adipose tissue dysfunction that is associated with increased inflammation and adipose tissue fibrosis, factors that may negatively impact stem/stromal cell survival. Thus, we hypothesize that HFD will negatively impact NG2⁺ pericyte relative quantity. Pericytes possess adipogenic capacity and differentiation may be stimulated as a result of HFD. Thus, adipogenesis may also lower NG2⁺ pericyte quantity. If true, NG2⁺ pericytes may express common surface markers associated with adipogenic lineage (Lin⁻CD29⁺CD34⁺Sca-1⁺ and PDGFR α ⁺) in response to HFD. We further hypothesize that these changes will be reversed with engagement in endurance training.

Alternative hypothesis: Inguinal adipose tissue was used so that fat pads would be available in both male and female mice. Since subcutaneous fat is less prone to inflammation, HFD may not result in adipose tissue dysfunction. In this case, it is possible that no change in pericyte quantity will be observed, nor changes in cell surface marker expression.

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CHAPTER 2: LITERATURE REVIEW

Obesity is a rapidly increasing condition and over one third (36%) of American adults are obese with BMI of 30 or higher [1]. In the obese condition, adipose tissue expands in order to allow for proper storage of the increased lipid input. Once lipid flux exceeds the adipocyte capability to hypertrophy or the adipose tissue capacity to expand, a complex chain of events take place which eventually result in adipose tissue dysfunction, lipid spillover [2-4] and lipotoxicity [5-6], and adipose tissue as well as systemic inflammation, which are likely to cause metabolic impairments, including type 2 diabetes [7-9].

2.1 Adipose Tissue Health

Rosen and Spiegelman [10] have recently stated that “...*adipocytes are perhaps the most vilified nonmalignant cell type in the body. Given that context, it has been easy to overlook the many benefits provided by healthy adipose tissue*”.

Considering that the main function of adipose tissue is regulating energy homeostasis through modulating lipid storage and release, a healthy adipose tissue will promote processes that ensure the balanced execution of these events including lipid storage during energy surplus (postprandially) and lipolysis during energy deficits (fasting). Several studies implicate circulating lipids in inducing lipotoxicity that underlies many of the deleterious consequences of obesity, from elevated inflammation to cardiomyocyte death [11,5]. However, lipodystrophic mice models that lack adipose tissue present a similar metabolic phenotype to that of obese mice, characterized primarily by insulin resistance [12-14]. In lipodystrophic and lipoatrophic mice, lipids are not stored in a designated depot, but rather lipids are deposited in other organs such as

skeletal muscle and the liver and impair insulin signaling which manifests as hyperglycemia [12,14].

In order to ensure adequate lipid storage capacity, adipose tissue is under constant remodeling and it is estimated that 8-10% of human adipocytes are replaced every year [10,15]. Adipose tissue remodeling is a highly controlled process which balances adipocyte death with adipogenesis, allowing the total adipocyte number to remain fairly constant during adulthood [15]. When exposed to chronic hypercaloric diet, adipose tissue expands via adipocyte hypertrophy, hyperplasia or both [7,8,16]. Adipose tissue expansion involves carefully orchestrated ECM remodeling, adipogenesis, and angiogenesis [9, 15-17]. In addition to adipocytes, adipose tissue consists of fibroblasts, mast cells, preadipocytes, immune cells (macrophages and T-cells) and cells associated with the vasculature, including endothelial cells, smooth muscle cells, and pericytes [17-19]. These different cell populations must work in concert to remodel the adipose tissue in order to increase lipid storage capacity and maintain adipose tissue health through balanced utilization of adipocyte hypertrophy and hyperplasia [17,9].

2.2 Adipose Tissue Mesenchymal Stem Cells (ADSCs)

Adult stem cells are maintained in a variety of tissue types for the purpose of regenerating injured tissue throughout the lifespan. Stem cells must fulfill two important criteria, the first is having the ability to self-renew via asymmetric cell division. During asymmetric cell division, a stem cell will give rise to a single daughter cell that will differentiate and replace lost tissue, while the other daughter cell will remain undifferentiated and return to quiescence. The second criterion is multipotency or the ability to differentiate into a variety of cell types [20-22].

Mesenchymal stem cells (MSCs) originate from the mesoderm during embryonic development and reside in nearly all tissues in the human body. MSCs have been isolated from bone marrow, skeletal muscle, adipose tissue and other organs as well [23]. Regardless of their origin MSCs express a variety of cell surface markers, including Sca-1 (restricted to murine model), CD29, CD90, CD105, and can readily differentiate into a variety of mesodermal tissue types. MSCs are resident in adipose tissue and easily differentiate into cells of mesenchymal lineage such as osteocytes, chondrocytes and adipocytes [16,20,24]. Currently, adipose-derived MSCs (or ADSCs) are considered a desirable tool in the development of stem cell therapies for a variety of diseases and conditions given the fact that subcutaneous adipose is easily accessible and extremely plastic.

Adipogenesis, the *de novo* formation of adipocytes from committed adipose tissue stem cells (preadipocytes) is necessary during development, as well as periods of excess nutrient intake. In the course of diet-induced obesity, once adipocyte death and inflammation start to abate, small newly formed adipocytes increase in quantity and are associated with improved metabolic profile [8]. Newly formed fat tissue was first discovered in the 1940s when transparent chambers were implanted in rabbit ears and adipocyte formation was directly visualized [16]. Since then, significant effort has been focused on identifying the origin of adipocytes and the molecular events that control adipogenesis [16,19,25].

Currently, ADSCs are believed to contribute a significant role in adipogenesis. However, due to the fact that investigators have used various strategies for isolation and characterization, there is no consensus in the field regarding the combination of cell surface markers required for identification of the preadipocyte [16, 26-31]. Despite this limitation, the majority of studies to

date suggest that preadipocytes reside in the microvascular niche in adipose tissue and possess a cell surface marker profile that is similar to MSCs [16, 23, 26, 32-33].

Adipogenesis is typically divided into two stages: determination and terminal differentiation [10, 34-35]. In the first phase, adipose stem cells undergo a series of intracellular and transcriptional events that promote lineage commitment and preadipocyte formation [10]. Terminal differentiation is the second phase in which preadipocytes develop into mature adipocytes and acquire adipocyte characteristics (unilocular lipid droplet surrounded by a specific protein envelope – perilipin) [10, 34].

As mentioned above, there is some controversy and much confusion regarding identification and nomenclature of preadipocytes [16,21]. The most important criterion for identification of the adipose progenitor cells is its ability to differentiate into adipocytes *in vivo* [21]. One of the most important studies addressing this question was published in 2008 when Rodeheffer and colleagues isolated a specific population of progenitor cells from stromal vascular fraction (SVF) of murine adipose tissue [29].

Rodeheffer and colleagues used a sophisticated FACS strategy to eliminate lineage (Lin) cells from the SVF by negatively selecting for hematopoietic, erythroid and endothelial cell markers (CD45, Ter119 and CD31, respectively) and then purifying the stem cell population until a very small, homogenous population of adipose progenitors remained. Rodeheffer *et al.* injected the highly purified Lin⁻CD29⁺CD34⁺Sca-1⁺CD24⁺ population into lipodystrophic A-Zip mice in which a strong endogenous adipogenic stimulus exist. The injected cells restored adipose tissue and rescued the diabetic phenotype typical to these mice [29].

The repeated sorting strategy used in this study yielded several different subpopulations that possessed multi-lineage differentiation and adipogenic potential *in vitro*, however, only the

$Lin^-CD29^+CD34^+Sca-1^+CD24^+$ population formed adipocytes *in vivo* [29]. Adipogenic differentiation was until then (and largely today as well) demonstrated mostly *in-vitro*, the originality of Rodeheffer et al. study [29] was the demonstration of *in vivo* adipogenesis after injection of a homogenous population of adipogenic progenitors from luciferase reporter mice donors. Rodeheffer and colleagues were able to visualize non-invasively adipose tissue development by imaging luciferase activity in these animals [21].

Recently, with the use of a Cre-recombinase technique, Berry and Rodeheffer [27] characterized the adipogenic lineage and found that platelet-derived growth factor receptor α (PDGFR α) is an essential marker of adipogenic cells. They also determined that contrary to some studies that suggest a common progenitor population for adipogenic, endothelial and hematopoietic lineages [16], they do not converge [27]. In addition, the results from this study suggested that $Lin^-CD29^+CD34^+Sca-1^+CD24^-$ cells are committed preadipocytes derived from $Lin^-CD29^+CD34^+Sca-1^+CD24^+$ stem cells [27].

The slow process of cell surface marker identification and subsequent evolution of nomenclature is common in the field of stem cell biology. Zimmerlin and colleagues suggest that adipose tissue contains three distinct stem cell populations that express typical MSC markers (CD73, CD90 and CD105), within the perivascular niche: 1) endothelial progenitors ($CD45^-CD31^+CD34^+$), 2) supra-adventitial adipose stromal cells ($CD45^-CD31^-CD146^-CD34^+$), and 3) pericytes ($CD45^-CD31^-CD146^+$) [36]. Similar to Rodeheffer et al. [29] these cells are also Lin^- but further comparison is not possible since no information is provided regarding expression of the other important MSC or pericyte markers. Thus, the full extent to which these cells engage in adipogenesis is not clear.

2.3 Adipose Tissue Pericytes

Pericytes are mural cells that reside around capillaries and microvessels and share a common basement membrane with endothelial cells. Pericytes are present in brain, lungs, kidney, liver, pancreas, retina, skeletal muscle and adipose tissue [23]. Pericytes provide structure to vessels, assist in transport of materials across the BBB, and regulate vessel dilation [37]. Pericytes are clearly essential for maintenance of vascular function and integrity. However, recent studies suggest that pericytes also serve as multipotent stem cells that can differentiate into several cell types, including osteocytes, chondrocytes, myocytes and adipocytes [37]. Resident pericytes additionally serve as stromal cells that secrete paracrine factors that contribute to ECM remodeling and angiogenesis [37-39]. Therefore, the role for the pericyte in a wide variety of tissues appears to be expanding and now includes involvement in tissue regeneration and maintenance of whole tissue health.

Pericytes are traditionally identified by morphology and distinct localization surrounding blood vessels as detected by high power microscopy. However, several surface markers are currently used for the identification of pericytes. The most common markers are smooth muscle α -actin (α SMA), desmin, NG2, PDGFR β , RGS5 and Melanoma Cell Adhesion Molecule (M-CAM/CD146) [23,38,40]. However, due overlapping expression with other stromal cells, one must utilize a combined methodology of surface marker expression together with histology analysis of localization [40]. In an elegant study in which the stromal vascular niche had been maintained while adipocytes were removed, Tang *et al.* were able to verify pericyte localization in adipose [32]. Moreover, the authors identified a distinct pericyte marker combination that characterized adipogenic cells (α SMA, PDGFR β and NG2) [32].

Birbrair and colleagues have recently published several papers [41-43] describing and characterizing two newly discovered pericyte subpopulations in skeletal muscle. Both were associated with microvessels and expressed common pericyte markers, including NG2, PDGFR β , and CD146 [41-43]. After selection based on the expression of NG2, pericytes were separated into two distinct populations using Nestin (neural stem cell marker). A double transgenic reporter mouse was used to obtain cells for further analysis, created by cross breeding one line that expresses DsRed-T1 fluorescent protein on the NG2 promoter and another line that expresses GFP on the Nestin promoter [41-42].

Type 2 pericytes present a myogenic profile, whereas Type 1 pericytes express PDGFR α (adipogenic progenitor marker) and undergo adipogenic differentiation *in vitro* and *in vivo* when transplanted into skeletal muscle after glycerol-induced injury [41]. Additionally, when exposed to TGF β *in vitro*, Type 1 pericytes become fibrogenic and differentiate into FSP1⁺ fibroblast like cells which express collagen 1. Finally, when injected into barium chloride injured skeletal muscle, Type 1 pericytes secrete collagen and contribute to scar tissue formation [43]. Currently, the extent to which these pericyte subpopulations exist in adipose tissue and engage in adipogenesis is unknown.

2.4 Adipose Stem Cells in Obesity

Obesity is associated with adipose tissue inflammation and fibrosis, which is more prevalent in visceral compared to subcutaneous adipose tissue [8, 44]. Stem cells are sensitive to a variety of cues in the microenvironment and survival is often compromised in the presence of both inflammation and fibrosis [45]. Not many studies investigate the impact of obesity on adipose stem cell quantity, the two studies that do exist report no change in adipose stem cell content in response to obesity [46-47]. The impact of obesity on stem cell function is dependent

upon the adipose depot localization. For example, adipose stem cells extracted from visceral depots (epididymal in mice or omental in humans) inherently demonstrate poor adipogenic differentiation potential and reduced proliferation compared to stem cells obtained from subcutaneous depots [31,48-49]. Interestingly, studies suggest that obesity can negatively impact adipose stem cell function (i.e. differentiation and transcriptional activity) [47, 50]. However, stem cell stromal capacity is rarely assessed in studies and future studies are necessary to address this important question.

The full impact of obesity on adipose stem cell quantity and function remains unknown. Some studies suggest obesity may exhaust the capacity for preadipocytes to engage in self-renewal [16] and/or negatively impair paracrine secretion [9, 16, 50]. However, it is not possible to make solid conclusions regarding stem cell function due to limitations in identification of cell types across different tissues. [16,28,39].

2.5 Exercise and Adipose Health

The main factors that contribute to adipose tissue dysfunction in the context of obesity include the following: increased FFA influx, adipocyte hypertrophy and hypoxia, endoplasmic reticulum (ER) and oxidative stress, adipose tissue fibrosis and inflammation. In contrast, exercise training has been reported to suppress adipose tissue inflammation [51-53], alter macrophage polarization towards M2 phenotype [52], and improve cellular oxidative homeostasis by increasing the resistance to oxidative stress [51]. Additionally, exercise assists in the reduction of adiposity [53]. Exercise promotes systemic changes that attenuate the metabolic co-morbidities of obesity [51, 53-54]. Exercise training attenuates insulin resistance, reduces blood pressure and improves blood lipid profile [51].

Although they did not investigate the interaction between adipose stem cells and exercise, Gollisch and collaborators did evaluate the effects of exercise on subcutaneous and visceral adipose tissue in obese rats [55]. Four weeks of voluntary wheel running completely prevented the high fat diet associated increase in fat pad weight, adipocyte size and adipocyte number in both fat pads.

Hypoxia markers are also attenuated by exercise in obese rats. Lactate levels and VEGFa gene expression were used to evaluate hypoxia in epididymal and inguinal fat pads of obese rats [56]. Disanzo and You found that 8 weeks of treadmill exercise resulted in reduced lactate and increased VEGFa mRNA which suggests that exercise might attenuate adipose tissue hypoxia. Disanzo and You however conclude that these effects were depot-dependent and were significant only in the epididymal pads [56]. Kawanishi and colleagues used a 16 weeks long model of concomitant high fat feeding with treadmill exercise to demonstrate quite effectively the attenuation of adipose tissue fibrosis with exercise [57]. Histological analysis of epididymal fat pad clearly demonstrated increased Sirius Red, as well as Trichrome staining, in obese mice, an observation that was significantly attenuated by exercise training. Pro-fibrotic marker (col1, col3, TGF β and Timp1) gene expression was increased with obesity and attenuated with exercise [57].

FFA influx, oxidative stress, fibrosis and inflammation negatively impact adipose stem cell quantity and function. However, the extent to which exercise training may mitigate these factors in adipose stem cells is not known. We could not find studies that addressed the interaction between adipose stem cells and the adipose microenvironment in the context of obesity and exercise training. Although Sakurai *et al.* [58] reported that exercise training reduced SVF cell adipogenesis and lipid accumulation *in vitro* and may directly impact preadipocyte function, it is impossible to interpret from these findings in what manner exercise will affect

adipose stem cells in obese mice since Sakurai and colleagues did not challenge the mice with a high fat diet.

2.6 Conclusion

Evaluating adipose stromal cell response to obesity is of great importance. Adipose tissue is a preferred site for stem cell harvesting to use in research and in therapy. Whenever donors may be obese, their ADSC or pericyte quantity and function might be impaired and a simple exercise training intervention might potentially restore them. However, currently, very little is known regarding ADSC and pericyte response to HFD and exercise. The overall goal of this dissertation is to fill this gap in knowledge.

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CHAPTER 3: DIET-INDUCED OBESITY REGULATES ADIPOSE-RESIDENT STROMAL CELL QUANTITY AND EXTRACELLULAR MATRIX GENE EXPRESSION

3.1 Abstract

Adipose tissue expansion during periods of excess nutrient intake requires significant turnover of the extracellular matrix (ECM) to allow for maximal lipid filling. Recent data suggest that stromal cells may be a primary contributor to ECM modifications in visceral adipose. The purpose of this study was to investigate the capacity for high fat diet (HFD)-induced obesity to alter adipose-derived stromal cell (ADSC) quantity and ECM gene expression, and determine the extent to which exercise training can mitigate such changes. Male C57BL/6J mice were placed on control or HFD for 8 wk prior to and following initiation of a 16 wk treadmill exercise program. ADSCs (Sca-1⁺CD45⁻) were isolated from epididymal adipose tissue and mRNA was evaluated using high throughput qPCR. Stromal cells were also obtained from skeletal muscle (MDSC). While resulting in a total increase in ADSC quantity, HFD decreased the relative quantity of ADSCs and markedly altered gene expression related to ECM remodeling (Col1 α 1, MMP2, MMP9, Timp1). Exercise did not reverse these changes. MDSCs were minimally altered by HFD or exercise. Overall, the data from this study suggest that ADSCs increase in quantity and contribute to adipose ECM remodeling in response to obesity, and exercise training does not significantly impact these outcomes.

3.2 Introduction

Excess nutrient availability results in deposition of lipid into preexisting adipocytes (adipose hypertrophy) or newly formed adipocytes (adipose hyperplasia). Adipocyte filling or preadipocyte expansion, however, cannot occur without supportive changes to the microenvironment, including extracellular matrix (ECM) and vascular remodeling [1-4]. The stromal vascular fraction (SVF) is a heterogeneous mixture of non-adipocyte cells in the adipose tissue that allow for tissue plasticity. The SVF includes immune cells, fibroblasts, preadipocytes, and important vascular-associated stem cells, including endothelial progenitor cells (EPCs) (CD45⁻CD31⁺CD34⁺), adipose-derived stromal cells (ADSCs) (predominantly mesenchymal stem cells (MSCs)) (CD45⁻CD31⁻CD146⁻NG2⁻), and pericytes (CD45⁻CD31⁻CD34⁻CD146⁺NG2⁺) [4-5]. The complex cell-cell interactions that occur within the SVF to allow for appropriate tissue remodeling and expansion during conditions of healthy weight gain or obesity are not fully understood.

Stem cell antigen-1 (Sca-1) is a glycosyl phosphatidylinositol-anchored cell surface protein that was originally used as a marker to identify stem cells from bone marrow in the mouse, and is subsequently expressed by a variety of murine stem and progenitor cells in multiple tissues, including MSCs [6-7]. Recent studies suggest that ADSCs and adipocyte progenitors express Sca-1 [8-10] and that the lineage negative (Lin⁻) fraction (CD45⁻CD31⁻Ter119⁻) in white adipose tissue specifically represents MSCs. Sca-1^{high} stromal cells extracted from subcutaneous and visceral fat express a gene signature that reflects significant contribution to the synthesis and degradation of ECM molecules, including matrix metalloproteinases (MMPs) [11], and widespread downregulation of MSC gene expression is observed in Zucker diabetic fatty rats [12]. Interestingly, Sca-1^{-/-} mice display extensive skeletal muscle fibrosis

following injury as a result of a deficiency in MMP activity [13] and develop insulin resistance and elevated blood glucose in response to a HFD [14]. Thus, these studies suggest that ADSCs provide an essential role in adipose tissue remodeling, and engagement of ADSCs in this event may be impaired with obesity as a result of long-term high fat feeding. However, to our knowledge, the impact of long-term HFD-induced obesity on ADSC quantity and gene expression has not been evaluated.

Endurance exercise training results in adipose lipolysis and attenuation of fibrosis [15] and inflammation [16-17] in adipose and skeletal muscle, independent of weight loss. Thus, participation in an endurance training program is a well-established recommendation for individuals diagnosed with Type 2 diabetes. Currently, the impact of endurance training on stromal cell quantity and function in both adipose and skeletal muscle has not been investigated, particularly in the context of obesity. We have previously established that muscle-derived MSC (mMSC) relative quantity is increased in skeletal muscle in response to an acute bout of eccentric exercise, and that mMSC transplantation can facilitate improvements in myofiber growth and strength in response to training [18]. Thus, we speculate that resident stromal cells may provide the basis for positive changes in the structure and function of a variety of tissues, including both skeletal muscle and adipose, observed as a result of exercise.

In the present study, we hypothesized that ADSC quantity would be reduced [12], yet expression of genes related to ECM would be enhanced with HFD-induced obesity to sustain adipocyte growth. We also predicted that a 16 week endurance training program would prevent adipocyte growth and concomitant changes in ADSC quantity and gene expression. Using the same rationale, we hypothesized that muscle-derived stromal cell (MDSC) ECM gene expression

would be elevated in skeletal muscle following endurance training to facilitate structural remodeling.

3.3 Materials and Methods

Animals

Three month old male C57BL/6J mice (n=20) were purchased from Jackson Laboratories (Bar Harbor, ME) and were group-housed (3-4 mice per cage). Mice were kept on a 12 hr dark/light cycle (lights on 07:00 to 19:00 h) in a pathogen free, temperature-controlled facility and fed *ad libitum*. For the *in vitro* experiment, three male mice (mixed genetic background, SJL x C57BL/6) were used from our breeding colony. These mice were fed standard chow (Harlen-Teklad, 13% calories from fat). National Institutes of Health guidelines for the care and use of laboratory animals were strictly followed, and all experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Design

Mice were randomly assigned to one of four groups: control diet-sedentary (no exercise) (Con-Sed, n=5), control diet-exercise (Ex) (Con-Ex, n=5), high fat diet-sedentary (HFD-Sed, n=5) or HFD-exercise (HFD-Ex, n=3). Five animals were originally assigned to HFD-Ex, but two died immediately before study completion and were omitted from the analysis. All diets were purchased from Research Diets Inc. (New Brunswick, NJ) and consisted of 10% or 60% of the calories from fat (OpenSource Diets D12450B or D12492, for Con or HFD respectively). Nutrient composition of these diets is matched and reported on the Research Diets website. Mice were fed specialized diets for 8 weeks, and then either remained sedentary or were subjected to a 16 week progressive aerobic exercise program. The original diets (Con or HFD) were maintained throughout the study. At the end of 24 weeks, 36 hr after the last exercise bout, mice were

euthanized by CO₂ asphyxiation, tissues were harvested, and stem cells were isolated from epididymal fat pads and gastrocnemius-soleus muscle complexes. A graphical illustration of study design is presented in Figure 3.1.

Progressive aerobic exercise training

Exercise training was conducted on a motorized treadmill (Jog-a-Dog, Ottawa Lake, MI) for 60 min/day at 12-17 m/min, 5% grade, 5 days/week, for 16 weeks. Mice were introduced to treadmill exercise for a week, gradually running for 10-60 min at 10-12 m/min. Running speed was then increased every few weeks until the last two weeks of the study when running speed was 17 m/min. All animals complied with the exercise protocol. To control for stress associated with the training protocol, non-exercised control animals were exposed to similar noise and handling.

Hind limb grip strength measurement

Grip strength was measured using a hind limb pull bar on a 1027DM grip strength meter (Columbus instruments, Columbus, OH). Measurements were taken three weeks before the end of the study (wk 21). All mice were habituated to the procedure for 3 consecutive days followed by a day of rest before being subjected to grip strength measurement (on the fifth day). To assess strength, mice were secured by their scruff and tail and allowed to grip the pull bar using their hind limbs. The tester then gradually pulled the mouse back horizontally until the mouse grip was released. After 10 successful trials were performed only the 5 middle trials were used to record grip force in grams. The highest of these five values was used as peak force and the average of the five values was used as average force. Body weight was measured before each grip strength test and was used to express grip strength relative to weight (grip strength (g) divided by body weight (g)). All measurements were performed by the same evaluator.

Plasma analyses

Fasting plasma was collected in lithium-heparin coated conical tubes (Microvette CB300, Sarstedt, Nümbrecht, Germany) from the submandibular vein after 6 hr fasting, 5 days before the end of the study. Samples were centrifuged for 15 min (2000g at 4 °C) and were stored in -80 °C until analysis. Plasma glucose was measured using a colorimetric assay (Cayman chemical, Ann Arbor, MI) and insulin was measured using an Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL), both according to the manufacturer instructions. The homeostasis model assessment method (HOMA-IR) was used to evaluate insulin resistance utilizing the following formula: Fasting glucose (mmol/l) * Fasting insulin (μU/ml) / 22.5 [19]. Systemic inflammation was evaluated using a Mouse Serum Amyloid-A ELISA kit (Alpco diagnostics, Salem, NH). Colorimetric and ELISA assays were analyzed using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT).

Stromal cell isolation

Stromal cell isolation was performed under a laminar flow hood using sterile technique as previously reported [20]. In brief, both epididymal fat pads and both gastrocnemius-soleus complexes were dissected from each individual mouse and extensively minced in PBS and subjected to enzymatic digestion in 0.2% Type I Collagenase (Worthington Biochemical Co., Lakewood, NJ) for 45 min with repeated trituration. After adding the inhibition solution (20% FBS in HBSS) the samples were spun for 5 min at 450 g and filtered through a 70 μm strainer. The cells were then blocked with anti-mouse CD16/CD32 antibody (eBioscience, San Diego, CA) for 10 min on ice to prevent non-specific Fc receptor mediated binding. Following the blocking step, cells were incubated with fluorescent-conjugated antibody cocktail (Anti Sca-1-PE, 600 ng/10⁶ cells and anti CD45-APC, 300 ng/10⁶ cells, eBioscience, San Diego, CA) diluted

in 2% FBS in PBS for 1 hr on ice, followed by 2 washes in 2% FBS in PBS. Fluorescence activated cell sorting (FACS) was performed on an iCyt Reflection System (Carle Hospital, Urbana, IL) and Sca-1⁺CD45⁻ cells were collected in RLT lysis buffer (Qiagen, Valencia CA) for gene expression analysis or in high glucose Dulbecco's modified eagle's medium (DMEM), 10% FBS, 5µg/mL gentamycin (growth medium) for *in vitro* experiments.

RNA extraction, cDNA synthesis and preamplification

Cell lysates were subjected to RNA extraction using RNeasy Micro kit (Qiagen, Valencia CA), following the manufacturer instructions. Flash frozen epididymal fat pads were lysed in QIAzol lysis reagent and were subjected to RNA extraction using RNeasy Lipid Tissue Mini kit (Qiagen, Valencia CA). RNA quantification was completed in duplicate on a Take-3 application plate using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY) with starting RNA concentrations of 500 ng for tissue or 25 ng for cell lysates. Preamplification of Sca-1⁺CD45⁻ cell cDNA was performed using TaqMan PreAmp Master Mix (Life Technologies, Grand Island, NY). This technique allows for unbiased targeted amplification of genes using a primer pool of up to 100 Taqman primers, resulting in a high quantity of primer-specific amplified cDNA from minimal RNA (10-20 ng) [21]. Primer pool consisted of 32 inventoried Taqman primers, diluted to 0.2X in Tris-EDTA buffer. After mixing the PreAmp reagent (25µl per reaction) with the primer pool (12.5 µl per reaction) and sample cDNA (12.5 µl), individual reactions were amplified for 14 cycles in a thin-walled 0.2 ml PCR tube using a thermo-cycler (ABI Geneamp 9700, Life Technologies, Grand Island, NY).

Quantitative PCR

Quantitative PCR was performed on an Applied Biosystems 7900HT Fast RT PCR machine using inventoried Taqman primers purchased from Applied Biosystems (Life Technologies, Grand Island, NY). Primer information and assay ID numbers are provided in Table 1. Glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was used as the housekeeping gene and relative gene expression analysis was presented relative to the reference group (Con-Sed) using the $\Delta\Delta C_t$ method. All replicate C_t values were within 0.2 C_t units of each other.

In vitro sodium-palmitate assay

A single FACS-isolation was used to obtain a pool of adipose-derived Sca-1⁺CD45⁻ cells from 3 mice. Cells were seeded on 100 mm plastic culture dishes (1×10^6 cells per plate) for 8 days. Growth medium was changed every 3-4 days (see *Stromal Cell Isolation* for medium information). Cells were then seeded at equal density on laminin coated 6-well plates. Cells were allowed to adhere for 24 hr in growth medium. A previously published *in vitro* free fatty acid (FFA) assay was used [22] with modifications. Cells were incubated with 0.75 mM sodium-palmitate (5:1 FFA to protein molar ratio) in DMEM with 5% BSA (palmitate, n=3 wells) or 5% BSA in DMEM alone (vehicle, n=3 wells) for 3 days. Prior to application, palmitate was conjugated to BSA for 1 hr at 40°C with agitation (140 rpm). At the end of 3 days, media was removed, 350 μ l buffer RLT (Qiagen, Valencia CA) was added to each well and lysates were stored at -80°C until used for high throughput microfluidics qPCR. Images were obtained pre- and post-incubation, using a Zeiss AxioCam digital camera and Axiovision software at 20X magnification (Zeiss, Thornwood, NY, USA).

High throughput microfluidics quantitative PCR

RLT (Qiagen, Valencia CA) lysates from the *in vitro* experiment were subjected to RNA extraction and cDNA synthesis as mentioned above. qPCR was performed using a dynamic array integrated fluidic circuit (IFC) on a Biomark HD (Fluidigm, San Francisco, CA). This high throughput microfluidic qPCR system allows for a more accurate, highly repeatable analysis of multiple genes in multiple samples. Gene expression was assessed using inventoried Taqman primers (primer information and assay ID numbers are provided in Table 3.1). GAPDH was used as the housekeeping gene and relative gene expression analysis was presented relative to vehicle condition using the $\Delta\Delta C_t$ method. All replicate C_t values were within 0.2 C_t units of each other.

Sirius red staining

Adipose tissue was fixed in 4% formalin and embedded in paraffin. 8 μm sections were mounted on a microscope slide and were prepped for staining. Tissue sections were fixed in Bouin's solution and blocked in Fast Blue solution (0.1% Fast Blue RR Salt, 7 mM Magnesium Sulfate and 60 mM magnesium borate). Slides were immersed in Picro-Sirius Red solution (0.1% Sirius Red F3B in Picric Acid) and then quickly washed with acidified H_2O (0.5% Acetic Acid in dH_2O). After dehydrating the tissue with ethanol and xylene, cover slips were mounted with histological mounting medium (Permount, Fisher Scientific, Pittsburgh, PA). Collagen staining was visualized under light microscopy (20X) using an inverted microscope (Leica DMRXA2). Representative images were captured using a digital camera – AxioCam, and Axiovision software (Zeiss, Thornwood, NY).

Statistical analysis

All data are presented as means \pm standard error of the mean. Mixed model (within and between subjects) three-way ANOVA was used to investigate main and combined effects of diet

(HFD vs. Con), exercise (Ex vs. Sed) and tissue (adipose vs. muscle), on Sca-1⁺CD45⁻ stromal cell gene expression, followed by Tukey *post hoc* analysis. Two-way ANOVA followed by LSD *post hoc* analysis was used to investigate diet (HFD vs. Con) and exercise (Ex vs. Sed) main effects and diet by exercise interaction on adipose weight, skeletal muscle weight, ADSC and MDSC relative quantity, fasting insulin, fasting glucose, HOMA-IR, and cytokine gene expression in adipose tissue. Changes in body weight were analyzed using a three-way repeated measures ANOVA followed by LSD *post hoc* analysis to investigate main and combined effects of diet (HFD vs. Con) and exercise (Ex vs. Sed) over time (Pre, 8, 13, 21 and 24 wk). Student's T-test analysis was used to investigate the effect of palmitate compared to vehicle on ADSC gene expression. Before each analysis, distribution curve normality was verified (Shapiro-Wilk test, $p > 0.05$) and log transformation was used to correct non-normal distribution when needed. Statistical analysis (Two-way ANOVA, three-way repeated measures ANOVA and T-test) was performed using SPSS Ver. 20 (IBM, Chicago, IL) or (Mixed model three-way ANOVA) using SAS Ver. 9.3 (SAS Institute, Cary, NC). Differences were considered significant at $p \leq 0.05$.

3.4 Results

Long-term HFD results in obesity, low-grade inflammation and insulin resistance

Body weights increased steadily in all groups (time effect, $p < 0.05$) throughout the intervention. However, compared to control diet, 8 weeks of HFD resulted in a robust increase in body weight (diet effect, $p < 0.05$) and this difference was observed throughout the duration of the study (time by diet interaction, $p < 0.05$, Figure 3.2A). Similarly, compared to control diet, 24 weeks of HFD resulted in increased fasting insulin, fasting glucose and HOMA-IR (diet effect, $p < 0.05$, Figures 3.2D, 3.2E, and 3.2F, respectively). Finally, HFD resulted in adipose tissue inflammation and systemic inflammation, demonstrated by increased gene expression of

macrophage marker and classic cytokines: F4/80, TNF α and IL-10 (diet effect, $p < 0.05$, Figures 3.2G, 3.2H and 3.2I, respectively), and increased serum amyloid A (SAA) and spleen weight (diet effect, $p < 0.05$, Figures 3.2J and 3.2K, respectively).

Exercise restores grip strength, but does not attenuate insulin resistance and adipose tissue inflammation

All mice adhered to the exercise program and ran a total of 3600 min over 16 week exercise intervention. A significant time by exercise interaction ($p < 0.05$) suggested that exercise contributed to reduced weight gain in HFD-Ex, but *post hoc* analysis of this interaction was not statistically significant ($p = 0.059$ and 0.094 for HFD-Ex vs. HFD-Sed in wk 21 and 24, respectively). HFD decreased grip strength compared to control diet (diet effect, $p < 0.05$), whereas progressive aerobic exercise improved grip strength in the HFD-Ex group (exercise effect, $p < 0.05$, Figures 3.2B and 3.2C). Systemic insulin resistance, inflammatory gene expression, and other markers of inflammation did not decrease in adipose tissue in response to training (Figure 3.2D-J). Interestingly, although exercise did not attenuate *adipose* inflammation or SAA, spleen weight (a proxy for systemic inflammation) was significantly different in the HFD-Con group compared to all other groups, including HFD-Ex ($p < 0.05$, Figure 3.2K). HFD-Ex spleen weight was similar to that of the control diet groups, suggesting that exercise had a beneficial outcome on *systemic* inflammation. The changes in tissue inflammatory gene expression with HFD prompted us to investigate stromal cell relative quantity and gene expression in response to HFD, exercise or both in combination.

Long-term HFD decreases relative- but increases total ADSC quantity

Despite the significant increase in body weight with HFD, average epididymal fat weight was not different between the groups (Figure 3.3A). Representative gating strategy plots are

presented for ADSCs and MDSCs (Figures 3.3B and 3.3E, respectively). Other than our population of interest (Sca1⁺CD45⁻, ADSCs) most of the remaining cells in the SVF were Sca1⁻. ADSC relative quantity decreased by 10% with HFD (diet effect, $p < 0.05$, Figure 3.3C) whereas ADSC total quantity increased from $3.9 \pm 0.45 \times 10^5$ and $4.0 \pm 0.23 \times 10^5$ cells in the Con-Sed and Con-Ex groups to $20.0 \pm 8.6 \times 10^5$ and $16.0 \pm 6.8 \times 10^5$ cells in the HFD-Sed and HFD-Ex groups, respectively (diet effect, $p < 0.05$). Exercise training did not result in a significant change in ADSC relative or total quantity. Gastrocnemius-soleus complex weight was not different between the groups (Figure 3.3D) and MDSC relative quantity (6-7% of mononuclear cells) did not change with HFD, exercise training or the combination of the two (Figures 3.3F). Similarly, MDSC total quantity was not different between the experimental groups ($0.9 \pm 0.01 \times 10^5$, $1.7 \pm 0.47 \times 10^5$, $1.0 \pm 0.19 \times 10^5$ and $1.0 \pm 0.23 \times 10^5$ cells in the Con-Sed, Con-Ex, HFD-Sed and HFD-Ex, respectively). We next investigated the mRNA expression of two common MSC cell surface markers: CD90 and CD105. In ADSCs, CD90 gene expression was not influenced by HFD or exercise (Figure 3.3G). ADSC CD105 gene expression, on the other hand, was significantly lower compared to MDSCs (tissue effect, $p < 0.05$, Figure 3.3H). HFD decreased, while exercise markedly increased CD105 gene expression in ADSCs (diet by exercise by tissue interaction, $p < 0.05$, Figure 3.3H). In MDSCs, cell surface marker gene expression was not changed by diet or exercise intervention.

ADSC ECM-related gene expression is altered following long-term HFD, but is not responsive to exercise

Stromal cells regulate tissue matrix remodeling via synthesis and secretion of structural proteins and enzymes that regulate the presence of ECM proteins. MDSC ECM gene expression was not significantly altered in response to HFD, exercise or both combined (Figures 3.4A-F). In

contrast, significant changes in ECM gene expression were readily apparent in ADSCs. Compared to MDSCs, connective tissue growth factor (CTGF) gene expression was reduced, and collagen 1 α 1 (Col1 α 1), matrix metalloproteinase 2 (MMP2), MMP9, tissue inhibitor of matrix metalloproteinase 1 (Timp1) and Timp2 gene expression were all increased in ADSCs (tissue effect, $p < 0.05$, Figure 3.4). Significant diet effects and tissue by diet interactions were noted for Col1 α 1, MMP2, MMP9 and Timp1 gene expression ($p < 0.01$). *Post hoc* analyses revealed Col1 α 1, MMP2 and Timp1 mRNA was increased in response to HFD ($p < 0.05$, Figures 3.4B, 3.4C and 3.4E, respectively). HFD decreased MMP9 gene expression ($p < 0.05$, Figure 3.4D).

In contrast to diet, exercise training did not significantly alter ADSC ECM-associated gene expression. Adipose tissue collagen was directly visualized using Sirius red staining to verify this observation. Adipose tissue collagen accumulation was apparent in response to HFD (Figure 3.5A). In addition, adipose tissue TGF β and collagen 1 α 1 mRNA were significantly increased with HFD (diet effect, $p < 0.05$, Figure 3.5B-C). However, exercise training did not reduce collagen accumulation or whole tissue gene expression.

Finally, vascular growth factor (VEGF α) and the transcriptional factor PGC-1 α promote angiogenesis and vessel remodeling necessary to support tissue growth. VEGF α mRNA levels decreased in MDSCs and increased in ADSCs in response to HFD (tissue by diet interaction, $p < 0.05$, Figures 3.4G-H). Exercise did not impact VEGF α or PGC-1 α gene expression in stromal cells from either tissue.

ADSC cytokine gene expression is enhanced relative to MDSCs, but is not affected by diet or exercise.

Sca-1⁺CD45⁻ stromal cells can modulate the activity of immune cells [23] and are an established source of cytokine production [23,24]. Pro-inflammatory cytokine (tumor necrosis

factor α (TNF α), interleukin-1 β (IL-1 β)), anti-inflammatory (IL-10, IL-1 receptor antagonist (IL-1ra)), and IL-6 gene expression was significantly higher in ADSCs compared to MDSCs (tissue effect, $p < 0.05$, Figures 3.6A-E). Transforming growth factor β 1 (TGF β 1) gene expression was not altered. ADSC and MDSC cytokine gene expression (TNF α , IL-1 β , IL-10, IL-1ra, and TGF β 1) was not significantly influenced by HFD or exercise (Figures 3.6A-D, 3.6F). ADSC IL-6 gene expression was decreased as a result of exercise (exercise by tissue interaction, $p < 0.05$, Figure 3.6E).

Palmitic acid alters ADSC gene expression *in vitro*

In vivo experiments demonstrated that HFD-induced obesity significantly increased ADSC ECM gene expression. We tested the hypothesis that direct exposure to the long chain FFA, palmitate, in the adipose tissue microenvironment influences ADSC gene expression. ADSCs maintained their fibroblast-like triangular morphology in culture in response to long-term incubation with palmitate (Figure 3.7A), but gene expression was significantly affected. Specifically, in response to palmitate, MMP9 and Timp1, as well as TGF β 1 and IL-6, gene expression significantly increased in response to palmitate ($p < 0.05$, Figure 3.7B and 3.7C, respectively).

3.5 Discussion

The purpose of this study was to investigate adipose- and skeletal muscle-resident Sca-1⁺CD45⁻ stromal cell responses to long-term HFD-induced obesity, alone or in combination with a 16 week endurance exercise program. Adult mice (3 month old at onset and 9 month old at the end of the study) were exposed to prolonged 60% HFD (total of 24 wk) to mimic an obesogenic lifestyle. Accordingly, HFD resulted in a doubling of average body weight (60 g HFD versus 30

g Con), as well as insulin resistance and fasting hyperglycemia (over 200 mg/dL). Long-term HFD-induced obesity did not affect stromal cell quantity or function in *skeletal muscle*, but significantly influenced stromal cell quantity and gene expression in *adipose tissue*. Most importantly, obesity resulted in striking and consistent changes in ADSC gene expression related to ECM remodeling, yet endurance training did not alter this profile.

Despite the significant increase in body weight and diabetic state with long-term HFD, average epididymal fat weight was not different between the groups. These results are similar to seminal work previously published by Surwit and colleagues almost 30 years ago [25] and more recently by Strissel *et al.* [3]. In the latter study, epididymal fat pad weight decreased while subcutaneous fat pad mass increased with sustained HFD during weeks 12 through 20 [3]. Hepatosteatorosis was also observed, which together with subcutaneous fat accumulation, may account for the increase in body weight [3]. It is not clear whether ADSC quantity or function is responsible for the lack of epididymal fat pad growth in the current study. ADSC relative quantity was reduced with HFD, and several factors may account for the decrease, including 1) SVF enrichment (fibroblasts, endothelial cells, and immune cells) [26], which is supported by our finding of increased total ADSC quantity in response to HFD, 2) cell death due to inflammation, and 3) enhancement of adipogenic differentiation. Our preliminary examination of early and late adipogenic marker gene expression (Ebf1, ZFP423, DLK1, and PPAR γ) did not suggest increased capacity for adipogenesis with HFD. Thus, further studies are necessary to elucidate the basis for the decrease in ADSC relative quantity with HFD in visceral adipose tissue and compare the responses between different depots.

Sca-1^{-/-} mice demonstrate deficiency in MMP activity and enhanced fibrosis in muscle [13]. In addition, these mice develop insulin resistance and glucose intolerance when exposed to

a HFD [14]. Recent studies suggest that Sca-1⁺ cells in the adipose SVF are MSCs that secrete proteins necessary for ECM and vascular remodeling, including membrane-associated and secreted collagenases, or MMPs [5,11]. In the current study, we demonstrate that Sca-1⁺CD45⁻ stromal cells display a gene signature that depicts strong capacity for secretion of ECM modulatory factors in response to long-term HFD. ADSC-mediated secretion of MMP2, Timp1, Timp2 and Coll1 α 1 may facilitate adipose tissue expansion and support, possibly in an attempt to maximize lipid filling during periods of excess energy intake. Adipocyte hypertrophy in the absence of connective tissue degradation and vascularization can result in cellular stress and hypoxia, eventually leading to necrosis and inflammation [4]. The marked decrease in MMP9 gene expression with HFD concur with published reports of decreased MMP9 levels in adipose tissue of insulin resistant rodents [27]. This may reflect failure of ADSCs to regulate immune cell activity [28] and angiogenesis [29] in the pathologically expanded adipose tissue. Similarly, the increases in VEGFa and PGC-1 α gene expression suggest an attempt to initiate vascularization necessary to prevent adipocyte hypoxia. The lack of change in cytokine gene expression (TNF α , IL-1 β , IL-10, IL-1ra, IL-6, and TGF β 1) in response to HFD provides evidence that ADSCs are not a primary cellular source of systemic inflammation. Overall, our gene expression analyses suggest that ADSCs serve as interstitial stromal cells that modify the extracellular environment to accommodate adipocyte growth.

A moderate intensity exercise training program did not appear to reverse the ADSC gene expression profile elicited by HFD, and this was in agreement with results for SAA, insulin resistance, plasma glucose, and adipose tissue TNF α mRNA levels. Exercise did decrease spleen weight, which is a proxy for systemic inflammation. This suggests some involvement of exercise in the modification of systemic inflammation. Other studies have reported suppression of

systemic and adipose inflammation with exercise in both animal and human studies [15-17, 30-32], yet in our review of animal studies, including the aforementioned, we noted that the mice were significantly younger at study completion (2-4 month old versus 9 month old in the current study). We purposefully used older mice to mimic adult-onset weight gain and susceptibility to the diabetic condition. Although the exercise protocol we used was longer and more intense than commonly used in similar studies [15-17,30], it is possible that our paradigm simply could not overcome tissue dysfunction as a result of the combination of advanced age at the initiation of the study and consumption of a diet that was exceptionally high in fat (60% kcal).

Lipotoxicity in adipose tissue is a complex process that is triggered by hypercaloric nutrition. As a result of the excess lipid flux into adipose tissue during the progression of obesity, adipocytes hypertrophy and die. This is accompanied by local macrophage infiltration, systemic inflammation and spillover of lipids into the circulatory system with subsequent deposition in distal organs such as liver and skeletal muscle [33]. Wu *et al.* [34] demonstrated that FFA treatment can reduce MSC multipotency and initiate adipogenesis when presented in culture. Thus, we hypothesized that the change in ADSC ECM gene expression in response to HFD was triggered directly by the exposure to FFA. While the increase in *Timp1* gene expression observed with HFD-induced obesity was replicated with FFA incubation, FFA exposure resulted in changes in *MMP-9* gene expression opposite those obtained *in vivo*. Similar to our preliminary findings and in support of previously published work [34-35] the expression of genes related to adipogenesis (*Ppar γ* , *DLK-1* and *ZFP423*) also did not change in response to FFA incubation (data not presented). Finally, in contrast to *in vivo* results, palmitic acid increased the expression cytokine expression, including *IL-6* and *TGF β ₁*. Therefore, these data suggest that alterations in

ADSC gene expression with HFD-induced obesity are not initiated as a result of direct exposure to FFA, but rather alternative cues provided by adipose growth.

Muscle-resident Sca-1⁺CD45⁻ cells were minimally responsive to HFD and/or endurance exercise. A significant reduction in VEGF gene expression was observed in MDSCs in response to HFD, yet no other changes were noted in MDSC relative quantity or the expression of genes related to ECM remodeling or inflammation. It is possible that transient changes in MDSC gene expression occurred with the onset of HFD and exercise training, yet these responses were no longer present post-training. A more extensive evaluation of the MDSC gene signature in response to HFD and endurance exercise is necessary to elucidate a novel role for MDSCs in events other than remodeling and growth, such as metabolic flexibility and fatigue resistance. Similarly, the MDSC transcriptome should be evaluated to validate a role for MDSCs in ECM remodeling and myofiber growth in response to strength training.

3.6 Conclusion

This study provides the first investigation of the epididymal adipose-resident Sca-1⁺CD45⁻ stromal cell response to long-term HFD, alone and in combination with progressive endurance exercise training in adult mice. As hypothesized, 24 weeks of HFD result in obesity, adipose inflammation, systemic inflammation and insulin resistance. HFD-induced obesity increase total ADSCs while decreasing ADSC relative quantity and substantially altered gene expression of ECM proteins, including MMPs, Timps, and collagen. Contrary to our hypothesis, exercise did not alter any of the changes observed. With a few exceptions (CTGF, VEGFa, TGFβ), mRNAs specific to ECM remodeling proteins and inflammatory cytokines were notably lower in Sca-1⁺CD45⁻ stromal cells isolated from skeletal muscle compared to adipose, and gene expression was predominantly unaffected by HFD diet and/or endurance exercise. The

mechanistic basis for changes in ADSCs remains unknown, but FFA exposure appears unlikely based on studies conducted *in vitro*. Overall, this study highlights a role for ADSCs in ECM remodeling as a result of high fat diet-induced obesity. We speculate that ADSCs remodel the connective tissue in a manner to allow for maximal adipocyte filling/expansion and prevent necrosis that can lead to systemic inflammation and Type 2 diabetes, yet this protective countermeasure becomes ineffective without an appropriate reduction in energy intake.

3.7 References

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3.8 Figure Captions and Figures

Figure 3.1

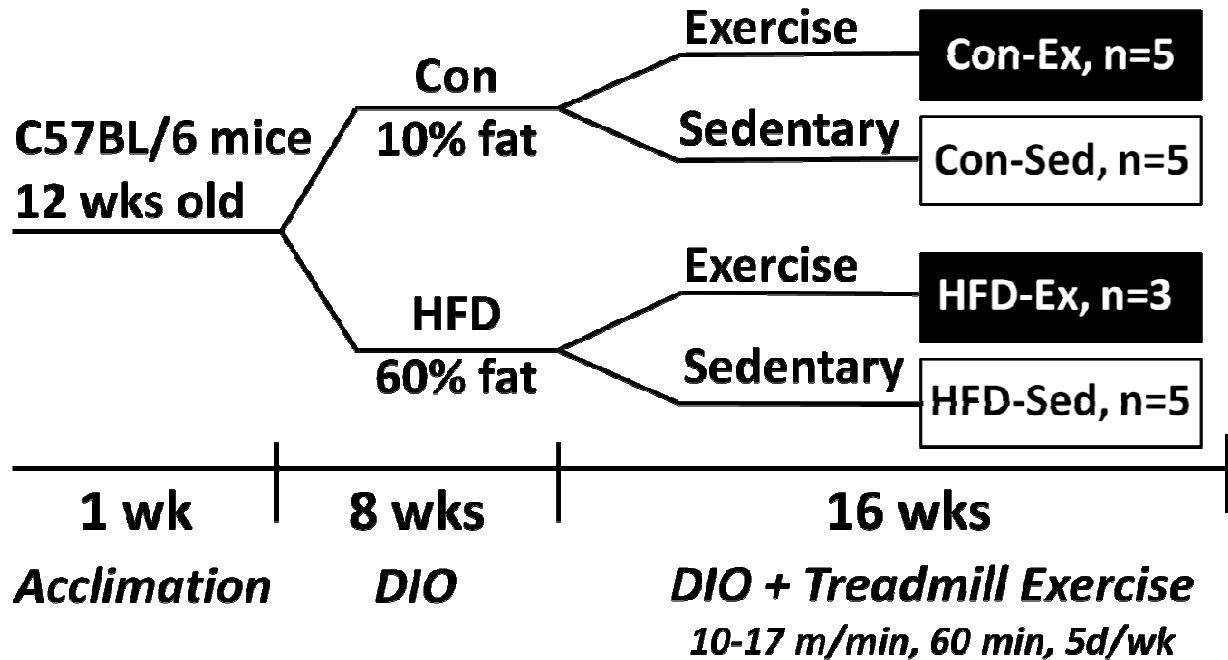


Figure 3.1 Study design. Mice were randomly assigned to one of four groups and were fed CON or HFD diets for 8 weeks. Mice then remained sedentary or were subjected to a 16 week progressive aerobic exercise program while maintaining their diets. Con, Control diet; HFD, High fat diet; Sed, Sedentary (no exercise); Ex, Exercise; DIO, Diet-induced obesity.

Figure 3.2

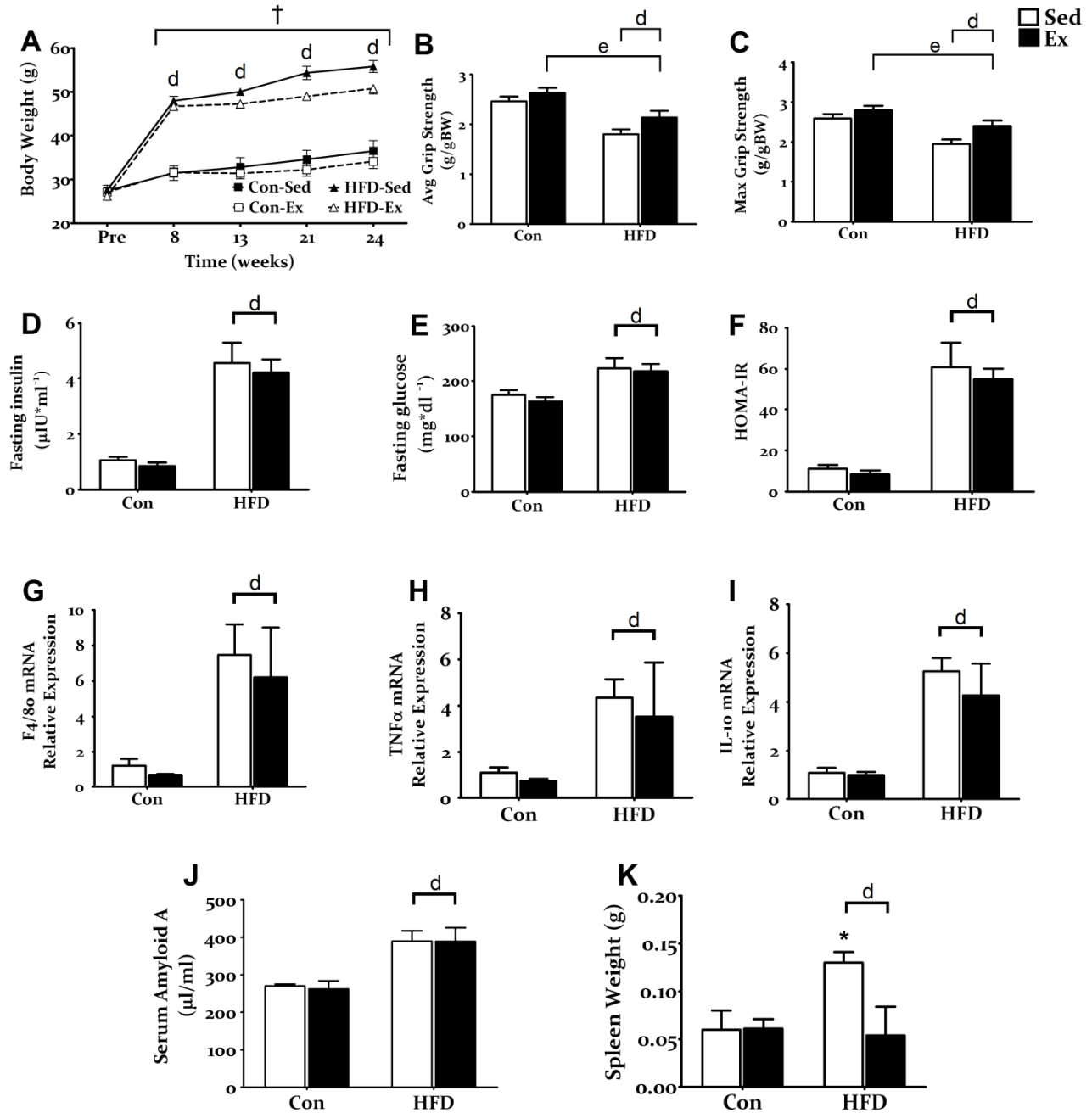


Figure 3.2 Long-term HFD results in obesity, insulin resistance and low-grade inflammation. Body weight (A), hindlimb grip strength (B, C), and spleen weight (K) were measured in grams. Fasting plasma insulin (D) and serum amyloid A (J) were measured using an ELISA kit, while fasting plasma glucose (E) was measured using a colorimetric assay. HOMA-IR (F) was calculated from fasting insulin and glucose values. Cytokine gene expression in adipose tissue (G, H, I) was examined using qPCR. Con-Sed, Con-Ex and HFD-Sed, $n=5$ and HFD-Ex, $n=3$. $^{\dagger}p\leq 0.05$ vs. pre-intervention (Pre); $^d p\leq 0.05$ vs. Con diet; $^e p\leq 0.05$ vs. Sed; $^* p\leq 0.05$ vs. Con-Sed. All values are mean \pm SEM.

Figure 3.3

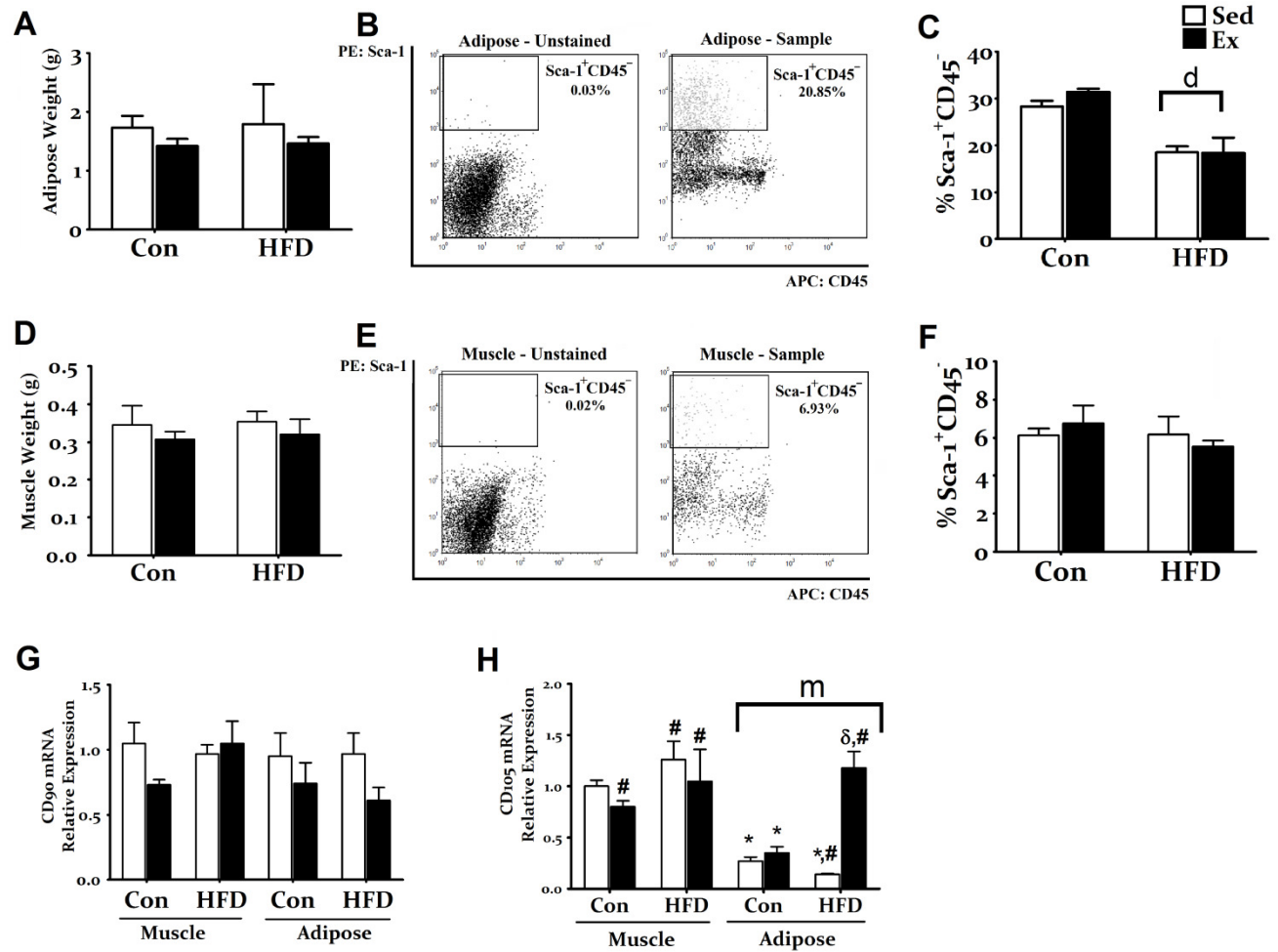


Figure 3.3 Long-term HFD decreases ADSC relative quantity. Sca-1⁺CD45⁻ cells were obtained from adipose (A-C) or skeletal muscle (D-F) of individual mice using FACS. Representative plots depicting gating strategy are presented (B, E). Tissue weight (A, D) was measured in grams. Sca-1⁺CD45⁻ cell relative quantity is presented for adipose (C) and for skeletal muscle (F). MSC marker (CD90 and CD105) gene expression (G, H, respectively) was examined in freshly collected ADSCs and MDSCs using qPCR. Con-Sed, Con-Ex and HFD-Sed, n=5 and HFD-Ex, n=3. ^dp≤0.05 vs. Con diet; ^mp≤0.05 vs. Muscle all groups; * p ≤ 0.05 vs. Muscle Con-Sed; # p≤0.05 vs. Adipose Con-Sed; ^δp≤0.05 vs. Adipose HFD-Sed. All values are mean ± SEM.

Figure 3.4

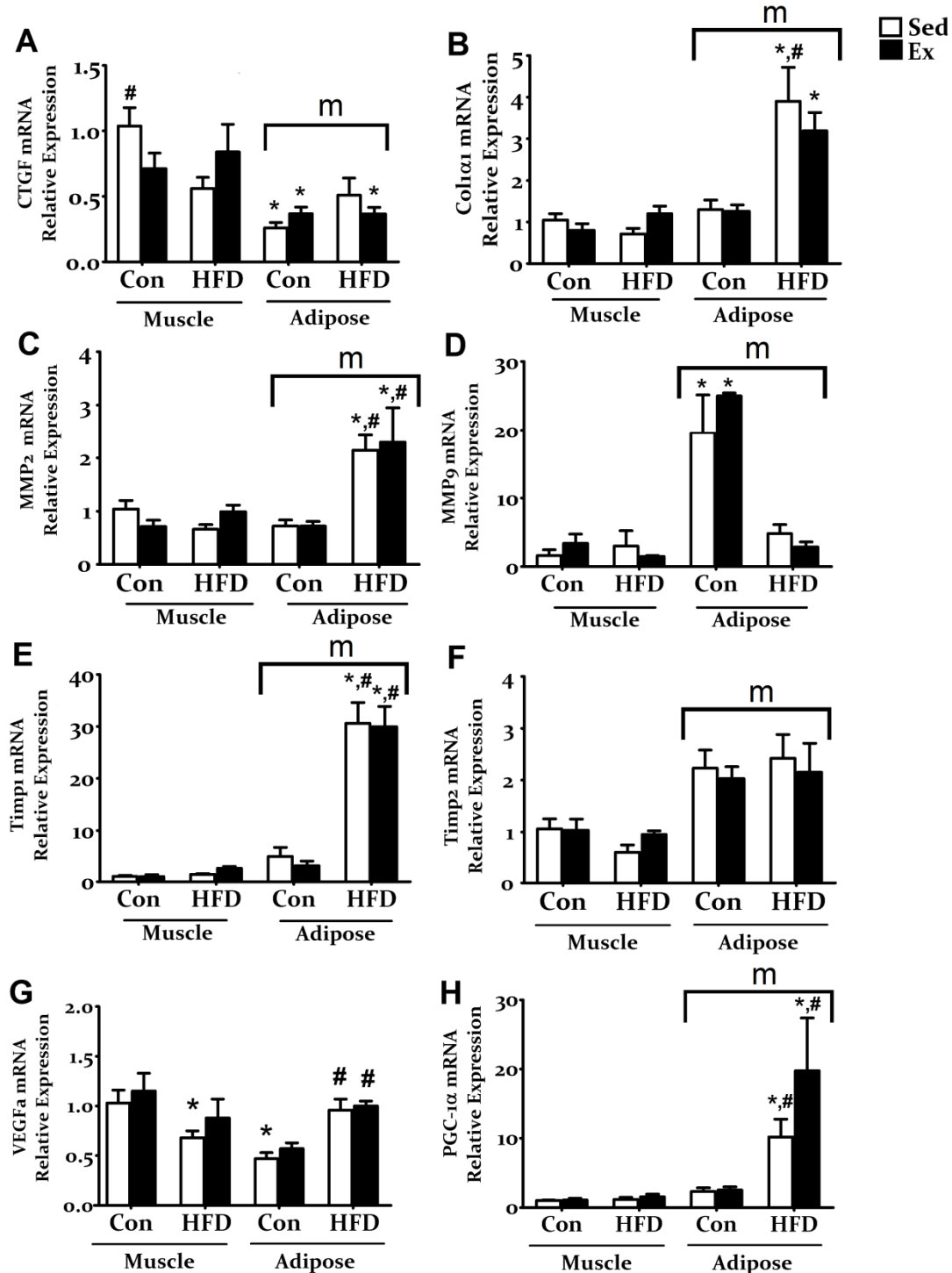


Figure 3.4 ADSC ECM-related gene expression is altered following long-term HFD, but is not responsive to exercise. CTGF, Col1α1, MMP2, MMP9, Timp1, Timp2, VEGFa and PGC1α (A-H) gene expression was examined in freshly collected ADSCs and MDSCs using qPCR. Con-Sed, Con-Ex and HFD-Sed, n=5 and HFD-Ex, n=3. ^mp≤0.05 vs. Muscle all groups; ^{*}p≤0.05 vs. Muscle Con-Sed; [#]p≤0.05 vs. Adipose Con-Sed. All values are mean ± SEM.

Figure 3.5

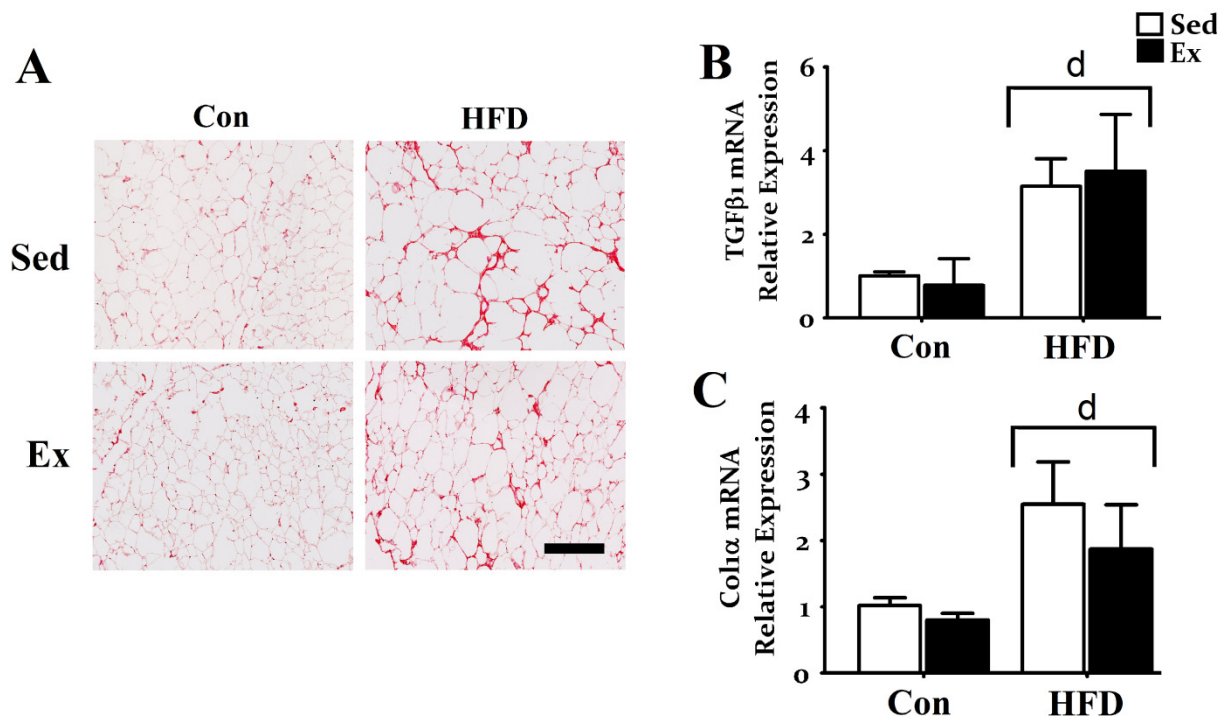


Figure 3.5 HFD results in adipose tissue fibrosis. Adipose tissue fibrosis was visualized in representative samples using histology (Sirius red, A) and assessed in all samples via gene expression (qPCR, B-C). ^d $p \leq 0.05$ vs. Con diet. All values are mean \pm SEM. A, scale bar=250 μ m.

Figure 3.6

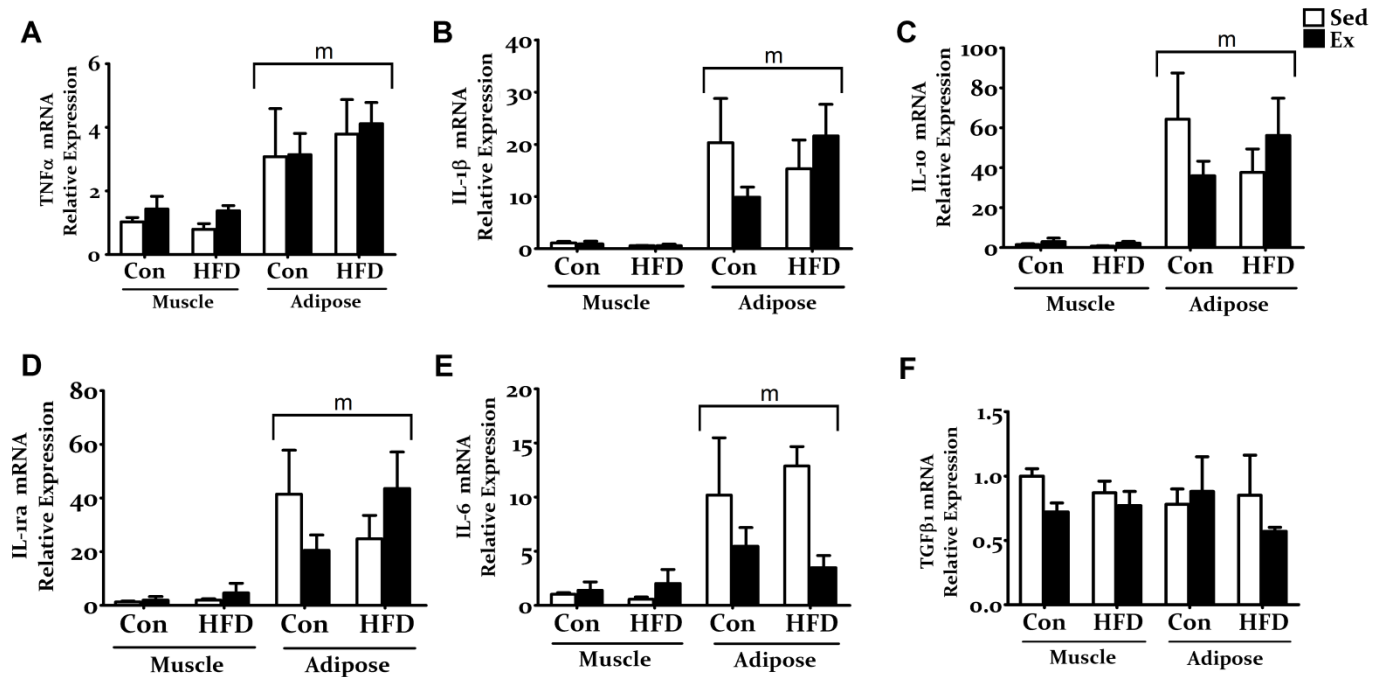


Figure 3.6 ADSC cytokine gene expression is enhanced compared to MDSC, but is not affected by diet or exercise. TNF α , IL-1 β , IL-10, IL-1ra, IL-6, TGF β 1 (A-F) gene expression was examined in freshly obtained ADSC and MDSC using qPCR. Con-Sed, Con-Ex and HFD-Sed, $n=5$ animals and HFD-Ex, $n=3$ animals. ^m $p < 0.05$ vs. Muscle all groups. All values are mean \pm SEM.

Figure 3.7

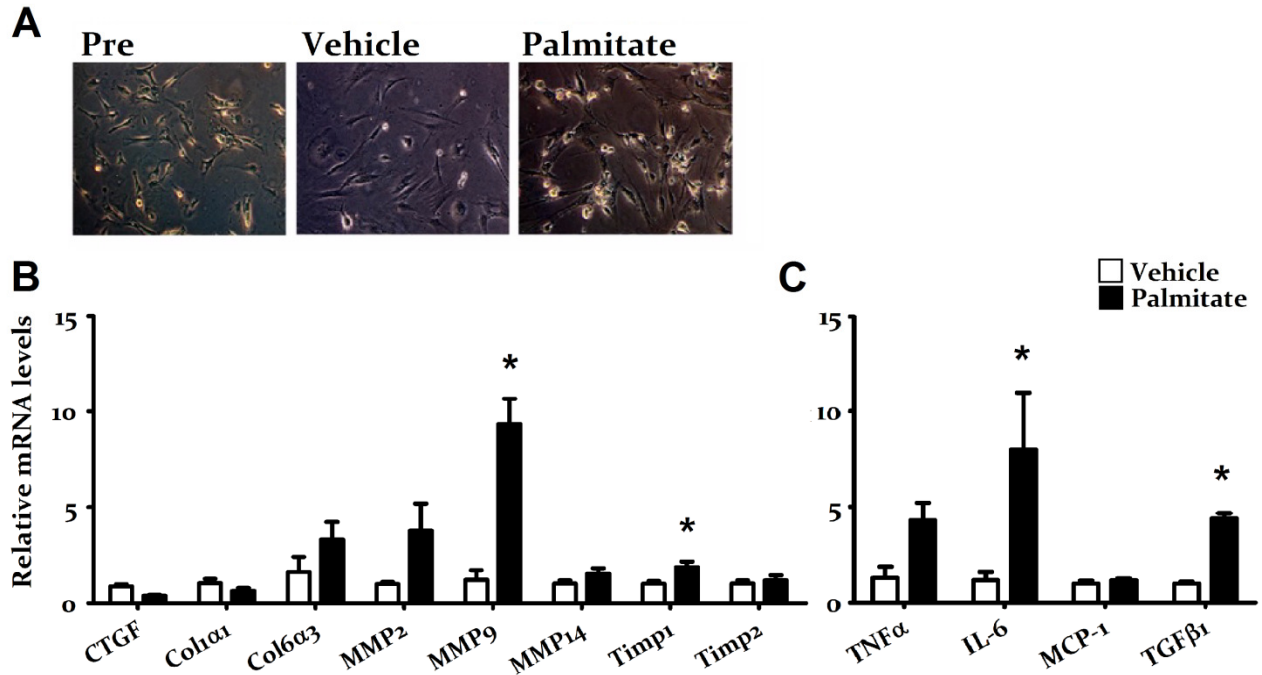


Figure 3.7 Palmitic acid alters ADSC gene expression *in vitro*. A single FACS was completed to obtain a pool of adipose-derived Sca-1⁺CD45⁻ cells from 3 mice. Cells were incubated with 0.75 mM sodium-palmitate (Palmitate, n=3) in DMEM with 5% BSA or 5% BSA in DMEM alone (Vehicle, n=3) for 3 days. Gene expression was analyzed using high throughput microfluidics qPCR. Bright-field images (A) shows no morphological changes. ECM remodeling protein (B) and cytokine (C) mRNA analysis shows altered gene expression in ADSCs in response to palmitate incubation. *p<0.05 vs. Vehicle. All values are mean ± SEM.

Table 3.1

List of Taqman gene expression assays		
	Gene/Alias	Assay ID
1	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Mm00484668_m1
2	CD90 (Thy 1)	Mm00493681_m1
3	CD105 / Eng (Endoglin)	Mm00468256_m1
4	Ppar γ -coactivator 1 alpha (PGC1 α)	Mm01208835_m1
5	Vascular endothelial growth factor a (VEGFa)	Mm01281449_m1
6	Connective tissue growth factor (CTGF)	Mm01192932_g1
7	Collagen 1 alpha 1 (Col1 α 1)	Mm00801666_g1
8	Collagen 6 alpha 3 (Col6 α 3)	Mm00711678_m1
9	Matrix metalloproteinase 2 (MMP2)	Mm00712992_m1
10	Matrix metalloproteinase 9 (MMP9)	Mm00442991_m1
11	Matrix metalloproteinase 14 (MMP14)	Mm00485054_m1
12	Tissue inhibitor of metalloproteinases 1 (Timp1)	Mm00441818_m1
13	Tissue inhibitor of metalloproteinases 2 (Timp2)	Mm00441825_m1
14	F4/80 (Emr1)	Mm00802529_m1
15	Tumor necrosis factor alpha (TNF α)	Mm00443260_g1
16	Chemokine (C-C motif) ligand 2 (CCL2)/ Monocyte chemotactic protein 1 (MCP1)	Mm00441242_m1
17	Interleukin 1 beta (IL-1 β)	Mm00434228_m1
18	Interleukin 1 receptor antagonist (IL-1rn)	Mm00446186_m1
19	Interleukin 10 (IL-10)	Mm00439614_m1
20	Interleukin 6 (IL-6)	Mm00446190_m1
21	Transforming growth factor beta 1(TGF β 1)	Mm01178820_m1

CHAPTER 4: EXERCISE ATTENUATES DIET-INDUCED CHANGES IN ADIPOSE-RESIDENT NG2⁺ PERICYTE QUANTITY

4.1 Abstract

Pericytes reside around blood vessels and regulate vascular remodeling, angiogenesis and extracellular matrix (ECM) turnover. In adipose tissue adipose progenitors share the same perivascular niche as pericytes. Recently discovered in skeletal muscle, NG2⁺Nestin⁻ (Type 1) pericytes are adipogenic. The extent to which Type 1 pericytes exist in adipose tissue and serve as adipose progenitors in response to high fat diet (HFD) has not been established. The purpose of this study was to investigate the capacity for HFD to influence adipose-derived NG2⁺Lin⁻ pericyte relative quantity and surface marker expression, and determine the extent to which exercise training can mitigate such changes. Male and female double transgenic NG2-DsRed x Nestin-GFP reporter mice (NG2xNes) were placed on control or HFD for 6 wk prior to and following initiation of a 12 wk treadmill exercise program. Adipose stromal cells were isolated from inguinal adipose tissue and surface marker expression (CD45, CD31, CD34, CD29, Sca-1, PDGFR α , CD90) was evaluated using multiplex flow cytometry. Type 2 pericytes were extremely rare in adipose tissue, thus further evaluation was performed on NG2⁺Lin⁻ pericytes. In males, HFD increased and exercise restored the quantity of NG2⁺Lin⁻ pericytes. No changes in pericyte quantity were noted in females. No changes in surface marker expression were detected in pericytes of male or female mice, but the overall quantity of NG2⁺Lin⁻ adipose precursors was extremely low. Overall, the data from this study suggest that in subcutaneous adipose in response to HFD, NG2⁺Lin⁻ pericytes do not directly contribute to adipogenesis but may promote changes

that support adipose expansion (e.g. angiogenesis, ECM remodeling), and exercise training may impact these outcomes in males.

4.2 Introduction

Pericytes encase capillaries and microvessels in various organs, including skeletal muscle and adipose tissue [1]. Pericytes are important regulators of vascular remodeling and angiogenesis, yet current studies suggest that pericytes are multipotent stem cells with capacity for differentiation into osteocytes, myocytes and adipocytes [2-3]. In addition, resident pericytes secrete a wide variety of paracrine factors that may be important for adipose tissue ECM remodeling [2-3]. Studies exploiting different methodologies, including fluorescent activated cell sorting (FACS), have identified pericytes residing in the microvascular niche of adipose tissue [1, 4-7]. However, a precise role for adipose-resident pericytes in the maintenance of tissue health during excess nutrient intake is unclear.

Pericytes and adipose progenitor cells reside in the perivascular niche of adipose tissue in both rodents and humans [4-6]. In response to adipogenic cues, adipose progenitors differentiate and form preadipocytes which mature into lipid-laden adipocytes. The extent to which pericytes contribute to the adipose progenitor pool in adipose tissue has not been established.

Recently, two distinct populations of pericytes were discovered in skeletal muscle [8-10]. The two populations were associated with microvessels and expressed common pericyte markers (NG2, PDGFR β , and CD146) [9-10]. Subpopulations were selected based on the expression of Neural/Glial antigen 2 (NG2, a chondroitin sulfate proteoglycan), a common pericyte marker, and were separated based on expression of Nestin (neural stem cell marker). Diverse functionality was established such that Type 1 pericytes (NG2⁺Nestin⁻) demonstrated adipogenic potential and Type 2 pericytes (NG2⁺Nestin⁺) engaged in myogenesis [8-9]. The extent to which

pericytes, particularly Type 1 pericytes [8-9], represent a reservoir of adipose progenitors or contribute a stromal role in adipose tissue health has not been established.

Obesity can stimulate adipose inflammation and fibrosis, factors that can compromise resident stem cell quantity and function. Conversely, endurance training can decrease adiposity, adipose tissue inflammation [11-13], fibrosis and oxidative stress [11]. Very few studies have addressed the interaction between adipose stem cells and the adipose microenvironment in the context of obesity and exercise training. Some studies report no change in adipose stem cell content in response to obesity [14-15], while we previously established that a prolonged high fat diet (HFD) can decrease adipose-derived stem/stromal cell (ADSC; Sca-1⁺CD45⁻) relative quantity in epididymal pads (Pincu *et al.* in progress, see Chapter 3 here). The extent to which adipose- derived pericyte quantity and function is influenced by HFD is not known, nor the extent to which these changes can be attenuated with exercise training.

Therefore, there were two primary goals to this study: 1) to identify and characterize Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes in adipose tissue, and 2) to evaluate changes in quantity and surface marker expression in these two populations in response to HFD and exercise. We hypothesized that both pericyte types would be localized in adipose tissue. In addition, we speculated that HFD would promote adipogenesis in the Type 1 pericyte, and that exercise would mitigate this response.

4.3 Materials and Methods

Generation of NG2-DsRed X Nestin-GFP Double Transgenic Mice

NG2-DsRed mice (STOCK Tg(Cspg4-DsRed.T1)1Akik/J, stock number: 008241) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used to establish a hemizygote breeding colony. NG2-DsRed mice express a red fluorescent molecule (DsRed.T1) under

the control of the mouse *Cspg4* gene. Thus, all cells that express NG2 protein emit a red signal when excited at 561nm. Nestin-GFP mice were purchased from Dr. Enikolopov's lab (Cold Spring Harbor, NY) and were used to establish a homozygote breeding colony. Nestin-GFP mice express a green fluorescent molecule (GFP) under the control of the mouse *Nes* gene. Thus, all cells that express the Nestin protein emit a green signal when excited at 488nm. The NG2-DsRed by Nestin-GFP (NG2xNes) double transgenic breeding colony was established in-house and the cross yielded close to 40% double transgenic pups per litter.

Genotyping for NG2-DsRed and Nestin-GFP

At 3 weeks of age, pups were weaned and transferred out of the parent cage. At this point ear or tail sample was collected and kept on ice until DNA extraction. DNA was extracted according to Truett *et al.* [16] with minor modifications, immediately after tissue collection. Briefly, 75 μ l of DNA lysis buffer (25 mM NaOH and 0.2 mM EDTA in ddH₂O, pH 12) was added and samples were incubated for 45min at 95°C with agitation. Samples were then cooled on ice for 5 min and 75 μ l of Neutralizing buffer (40 mM Tris HCl in ddH₂O, pH 5) was added. After 15 min an autoclaved pipette tip was used to remove all visible tissue remains and 2 μ l of DNA extract were used for polymerase chain reaction (PCR).

NG2-DsRed PCR reaction utilized 2 pairs of primers, one pair which detects both *Cspg4* and DsRed.T1 as a part of one 280bp construct (Forward 5`- TTC CTT CGC CTT ACA AGT CC – 3`, Reverse 5`- GAG CCG TAC TGG AAC TGG -3`) and a second pair which detects a ubiquitously expressed gene at 324bp (Forward 5`- CTA GGC CAC AGA ATT GAA AGA TCT -3`, Reverse 5`- GTA GGT GGA AAT TCT AGC ATC ATC C-3`) that was used as a technical internal control. The 10 μ l reaction contained 2 μ l DNA, 1X Flexi Go PCR buffer (Promega, Madison, WI), 2mM MgCl₂, 0.2mM dNTP mix (NEB, Ipswich, MA), 1 μ M of each primer,

0.03U/μl GoTaq Flexi DNA Polymerase (Promega, Madison, WI) and ddH₂O. Once the reaction was prepared in a 0.2ml thin-walled PCR tube it was incubated for 3 min at 94°C and then was cycled 35 times in the following conditions: 30 sec at 94°C, 1 min at 61°C, 1 min at 72°C. After the completion of thermal cycling (GeneAmp PCR System 9700, Applied Biosystems, Grand Island, NY), PCR products were held at 10°C until agarose gel electrophoresis was performed.

Nestin-GFP PCR reaction utilized one pair of primers (Forward 5'- ATC ACA TGG TCC TGC TGG AGT TC – 3' corresponding to the sequences from the GFP cDNA, and Reverse 5'- GGA GCT GCA CAC AAC CCA TTG CC -3' corresponding to the sequences from the 2nd intron of the nestin gene) which yielded a 510bp PCR product. The 25μl reaction contained 3μl DNA, 1X Flexi Go PCR buffer (Promega, Madison, WI), 1.5mM MgCl₂, 0.2mM dNTP mix (NEB, Ipswich, MA), 2μM of each primer, 0.05U/μl GoTaq Flexi DNA Polymerase (Promega, Madison, WI) and ddH₂O. Once the reaction was prepared in a 0.2ml thin-walled PCR tube it was incubated for 3 min at 94°C and then was cycled 35 times in the following conditions: 30 sec at 94°C, 1 min at 64°C, 1 min at 72°C. After the completion of thermal cycling (GeneAmp PCR System 9700, Applied Biosystems, Grand Island, NY), PCR products were incubated for 5 min at 72°C and held at 4°C until agarose gel electrophoresis was performed.

Electrophoresis was performed using 2% agarose gel (2g Agarose powder, 100ml 1X Tris-EDTA buffer with 0.5μg/ml EtBr; All reagents from Sigma-Aldrich, St. Louis, MO) in Tris-Borate-EDTA buffer at 100V for 35 min. The gel was then visualized under UV light using Bio-Rad ChemiDoc XRS system (Bio-Rad, Hercules, CA).

Animals

NG2-DsRed by Nestin-GFP (NG2xNes) double transgenic breeding colony was established in-house by crossing transgenic pups from NG2-DsRed colony with transgenic pups

from Nestin-GFP colony, both on a C57BL/6 genetic background. Mice were group-housed (3-4 mice per cage) on a 12 hr dark/light cycle (lights on 07:00 to 19:00 h) in a pathogen free, temperature-controlled facility and fed *ad libitum*. National Institutes of Health guidelines for the care and use of laboratory animals were strictly followed, and all experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Design

Five to 6 week old male and female mice were randomly assigned into one of four groups: control diet-sedentary (no exercise) (Con-Sed, n=4 females/ 3 males), control diet-exercise (Ex) (Con-Ex, n=4 females/ 3 males), high fat diet-sedentary (HFD-Sed, n=3 females/ 3 males) or HFD-exercise (HFD-Ex, n=4 females/ 3 males). All diets were purchased from Research Diets Inc. (New Brunswick, NJ) and consisted of 10% or 45% of the calories from fat (OpenSource Diets D12450B or D12451, for Con or HFD respectively). Nutrient composition of these diets is matched and reported on the Research Diets website. Mice were fed specialized diets for 6 weeks, and then either remained sedentary or were subjected to a 12 week treadmill exercise program. The original diets (Con or HFD) were maintained throughout the study. At the end of 18 weeks, 36 hr after the last exercise bout, mice were euthanized by CO₂ asphyxiation, inguinal fat pads were harvested and were subjected to stromal cell isolation. A graphical illustration of study design is presented in Figure 4.1.

Exercise Training

Exercise training was conducted on a motorized treadmill (Jog-a-Dog, Ottawa Lake, MI) for 60 min/day at 12 m/min, 5% grade, 5 days/week, for 12 weeks. Mice were introduced to treadmill exercise for a week, gradually running for 10-60 min at 10-12 m/min. All animals

complied with the exercise protocol. To control for stress associated with the training protocol, non-exercised control animals were exposed to similar noise and handling.

***In Vivo* Fluorescent Macro Imaging**

We utilized an *in vivo* macro imaging approach to record red and green fluorescent signals from NG2-DsRed- and Nestin-GFP-positive cells, respectively in exposed skin in the ears, paws and tails of NG2xNes mice. Throughout the procedure, mice were kept anesthetized under isofurane vaporizer anesthesia system (VetFlo, Kent Scientific, Torrington, CT). Mice were positioned on the imaging platform inside the *in vivo* dual wavelength dark box imaging system (Maestro, Cambridge Research & Instrumentation, Inc., Cambridge MA) and were imaged 3 times. First a bright field image was used to correct the mice position and to set the background overlay, next blue laser excitation was used (488nm) to detect GFP and finally a green/yellow laser excitation (561nm) was used to detect DsRed. In all images, a non-transgenic mouse occupied the first position to be used as a non-tagged auto-fluorescent control.

Fluorescent Micro Imaging

Cells suspensions were inspected under a Leica DMRXA2 fluorescent microscope (Leica Microsystems Inc., Buffalo Grove, IL) using the appropriate wave length filter. Images were obtained using a Zeiss AxioCam microscope-mounted digital camera and Axiovision software (Zeiss, Thornwood, NY, USA).

Stromal Cell Isolation and FACS/Flow Cytometry Analysis

Stromal cell isolation was performed under a laminar flow hood using sterile technique as previously reported [17]. In brief, excised inguinal fat pads or gastrocnemius-soleus complexes were extensively minced in PBS and were subjected to enzymatic digestion in 0.2% Type I Collagenase (Worthington Biochemical Co., Lakewood, NJ) for 45 min with repeated trituration.

After adding the inhibition solution (20% FBS in HBSS) the samples were spun for 5 min at 450g and filtered through a 70µm strainer. If no fluorescent-conjugated antibodies were used (only the fluorescent proteins DsRed and GFP were used), the cells were then washed twice in ice-cold 2% FBS in PBS and were analyzed as soon as possible. For multiplex flow cytometry assays cells were blocked with anti-mouse CD16/CD32 antibody (BD Biosciences, San Jose, CA) for 10 min on ice to prevent non-specific FC receptor mediated binding. Following the blocking step, cells were incubated with one or two separate fluorescent-conjugated antibody cocktails (*Panel 1*: Anti CD45-PE-Cy7, 1:100, anti CD31- PE-Cy7, 1:100, anti TER119-PE-Cy7, 1:100, anti CD29-BV421, 1:100, anti CD34-Alexa Fluor 647, 1:50, anti Sca-1-APC-Cy7, 1:100, and anti CD24-BV510, 1:100, BD Biosciences, San Jose, CA. *Panel 2*: Anti CD45-PE-Cy7, 1:100, anti CD31-BV510, 1:50, anti CD90.2-APC, 1:100, anti PDGFRa-BV421, 1:50, BD Biosciences, San Jose, CA and anti PDGFRb-APC-Vio770, 1:50, Miltenyi Biotec Inc., San Diego, CA) diluted in 2% FBS in PBS with 50µl Brilliant Stain buffer (BD Biosciences, San Jose, CA) for 40 min on ice, followed by 2 washes in 2% FBS in PBS and a final filtration through a 40µm strainer. Fluorescence activated cell sorting (FACS) was performed on an iCyt Reflection System (Carle Hospital, Urbana, IL) and NG2⁺Nestin⁻ and NG2⁺Nestin⁺ cells were collected in two separate tubes containing high glucose Dulbecco's modified eagle's medium (DMEM), 10% FBS, 5µg/mL gentamycin (growth medium). Flow cytometry was performed on LSRFortessa (BD bioscience, CA) using 500,000 events for each sample to allow for adequate detection of rare populations. Data were analyzed using FCS Express 5 Flow Cytometry Analysis software (De-Novo software, Glendale, CA). Unstained and single-stained controls were used for compensation. A primary gate based on physical parameters (forward and side scatter, FSC and SSC, respectively) was set to exclude dead cells and debris and fluorescence-

minus-one controls (FMO, contains the full antibody cocktail except for the controlled antibody) were used for gating specific marker-positive or negative populations of interest.

Tri-Lineage Differentiation Assays

Adipose-derived stromal cells were pooled from 2 mice and NG2⁺Nestin⁻ (Type 1) and NG2⁺Nestin⁺ (Type 2) pericytes were obtained via FACS. Pericytes were expanded for 14-20 days in growth medium and were not allowed to passage more than 3 times. Pericytes were seeded on 4-well chamber slides (15,000 cells/well for adipogenic differentiation, 8,500 cells/well for osteogenic differentiation, and 15,000 cells/well for myogenic differentiation assays) and after 2-4 days differentiation media were added. Adipogenesis and osteogenesis were induced using StemPro adipogenesis and osteogenesis differentiation kits, respectively (Gibco (Thermo Fisher Scientific), Waltham, MA), according to the manufacturer instructions. Myogenic differentiation was induced using 2% horse serum in DMEM with 5µg/mL gentamycin. All differentiation media were changed every 3-4 days and differentiation was assessed after 14 days for adipogenesis and myogenesis and after 21 days for osteogenesis.

Immunocytochemistry Assessment of Tri-Lineage Differentiation Assays

Differentiation medium was removed and cells were fixed for 30 min in 4% paraformaldehyde (PFA) and washed 3 times in DPBS. For adipogenesis evaluation cells were stained with 1:100 Bodipy 493/503 (Life Technologies, Carlsbad, CA) and 1:4000 DAPI cocktail for 30 min to stain intracellular lipids and cell nuclei, respectively. Cells were then washed 4 times in DPBS. Vectashield mounting medium (Vector laboratories Inc., Burlingame, CA) and cover slips were applied and slides were imaged. For myogenic evaluation cells were permeablized for 15 min with 0.25% Triton-X in DPBS. Cells were then washed and blocked for 1 hr with 10% horse serum in DPBS. Primary antibody against MF-20 (Developmental Studies

Hybridoma Bank, Iowa City, IA) was applied for 1 hr (1:50 in 1% horse serum in DPBS). After three 5 min washes, secondary TRITC conjugated anti-mouse antibody was applied for 1 hr (1:100 in 1% horse serum). Cells were then washed and DAPI was applied (1:20,000) to counter stain cell nuclei. Vectashield mounting medium (Vector laboratories Inc., Burlingame, CA) and cover slips were applied and slides were imaged. For osteogenesis evaluation cells were fixed with 10% PFA for 10 min, washed twice and stained with 2% Alizarin Red S in ddH₂O for 15 min (Life Technologies, Carlsbad, CA). Cells were then washed with water until runoff was clear. Vectashield mounting medium (Vector laboratories Inc., Burlingame, CA) and cover slips were applied and slides were imaged.

Blood Collection and Analysis

Fasting plasma was collected in lithium-heparin coated conical tubes (Microvette CB300, Sarstedt, Nümbrecht, Germany) from the submandibular vein after 6 hr fasting, 3 days before the end of the study. Samples were centrifuged for 15 min (2000g at 4°C) and were stored in -80°C until analysis. Plasma glucose was measured using a colorimetric assay (Cayman chemical, Ann Arbor, MI) and insulin was measured using an Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL), both according to the manufacturer instructions. The homeostasis model assessment method (HOMA-IR) was used to evaluate insulin resistance utilizing the following formula: Fasting glucose (mmol/l) * Fasting insulin (μU/ml) /22.5 [18]. All assays were analyzed using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT).

Statistical Analysis

All data are presented as means ± standard error of the mean. Two-way ANOVA followed by LSD *post hoc* analysis was used to investigate group (Con-Sed, Con-Ex, HFD-Sed,

HFD-Ex) and sex (Female vs. Male) main effects and group by sex interaction on body weight, adipose weight, fasting insulin, fasting glucose, HOMA-IR, and pericyte flow cytometry data. Before each analysis, distribution curve normality was verified (Shapiro-Wilk test, $p > 0.05$) and log transformation was used to correct non-normal distribution when needed. All statistical analysis was performed using SPSS Ver. 20 (IBM, Chicago, IL). Differences were considered significant at $p \leq 0.05$.

4.4 Results

Verifying Fluorescent Protein Expression

Since the NG2xNes mice were bred in house from two separate transgenic mouse colonies, it was imperative to verify transgenic expression. Hence, all NG2xNes pups were genotyped for NG2-DsRed (Cspg4-DsRed.T1) and for Nestin-GFP (Figure 4.2A-B). To provide additional verification, mice were subjected to *in vivo* dual wavelength dark box imaging (Maestro, Cambridge Research & Instrumentation, Inc., Cambridge MA). NG2-DsRed and Nestin-GFP expression was easily visualized in exposed skin in the ears, paws and tail (Figure 4.2C). This combined approach enabled us to discern double transgenic mice from single and non-transgenic mice. Next, we produced a single cell suspension of adipose stromal cells from NG2xNes mice and seeded them onto a plastic culture dish. We allowed the cells to adhere overnight and inspected the cells under a fluorescent microscope (Figure 4.2D). We could clearly detect red NG2-DsRed⁺Nestin-GFP⁻ (purple arrowheads), green NG2-DsRed⁻Nestin-GFP⁺ (green arrowheads) and yellow NG2-DsRed⁺Nestin-GFP⁺ cells, (yellow arrowheads).

Flow Cytometry Analysis of Pericyte Types in Skeletal Muscle and Adipose Tissue

The mice required for this study (NG2-DsRed x Nestin-GFP double transgenic) were uniquely created by breeding two lines of mice and were maintained in-house. Due to limited availability of the mice, we were forced to use only female mice to complete the first part of this investigation.

Compared to Type 2 pericytes (NG2⁺Nestin⁺), Type 1 pericytes (NG2⁺Nestin⁻) were more abundant in both skeletal muscle and adipose (2.6±0.09% and 2.8±0.5% in skeletal muscle and adipose, respectively) (Figure 4.3A-B). This difference was more pronounced in adipose

tissue, as Type 2 pericytes in adipose tissue were extremely rare (only $0.46 \pm 0.12\%$ of all stromal cells expressed both NG2 and Nestin).

We then verified the expression of additional pericyte markers in adipose-derived Type 1 and Type 2 pericytes. Platelet Derived Growth Factor Receptor (PDGFR) β and CD146 were both detected in Type 1 and Type 2 pericytes (Figure 4.3C-D). The proportion of PDGFR β^+ cells was 4-fold higher in Type 2 compared to Type 1 pericytes whereas CD146 $^+$ pericyte relative quantity was similar between the two pericyte types.

Further investigation of surface marker expression in Type 1 and Type 2 pericytes revealed expression of endothelial (CD31 $^+$, Figure 4.3E-F) and hematopoietic (CD45 $^+$, Figure 4.3G-H) markers within the NG2 $^+$ pericyte populations in skeletal muscle and adipose tissue. In skeletal muscle, $16.27 \pm 0.32\%$ of Type 1 pericytes and $34.15 \pm 3.52\%$ of Type 2 pericytes express CD31 while $40.36 \pm 0.24\%$ of Type 1 pericytes and $19.43 \pm 2.39\%$ of Type 2 pericytes are CD45 $^+$. In adipose tissue, CD31 expression within the pericyte population is similar to that in skeletal muscle; $12.00 \pm 4.00\%$ of Type 1 pericytes and $32.58 \pm 5.75\%$ of Type 2 pericytes express CD31. Compared to skeletal muscle, expression of hematopoietic marker CD45 in adipose-derived Type 1 and Type 2 pericytes is different, $34.40 \pm 0.40\%$ of Type 1 and $68.35 \pm 1.68\%$ of Type 2 pericytes are CD45 $^+$. It is important to note that since Type 2 pericytes are very rare, NG2 $^+$ Nestin $^+$ CD31 $^+$ and NG2 $^+$ Nestin $^+$ CD45 $^+$ cells in skeletal muscle and adipose tissue comprise less than 0.07% each of all stromal cells.

Tri-Lineage Differentiation Analysis in Adipose-Derived Type 1 and Type 2 Pericytes

In order to assess the lineage capacity of adipose-derived pericytes we subjected freshly isolated NG2 $^+$ Nestin $^-$ (Type 1) and NG2 $^+$ Nestin $^+$ (Type 2) pericytes to adipogenic, osteogenic and myogenic conditions *in vitro*. We found that both populations did not form multi-nucleated

myotubes and stained negative for myosin heavy chain (MF-20, Figure 4.4A). After exposure to osteogenic conditions, Type 1 pericytes differentiated and formed small clusters of Alizarin Red positive cells. However, Alizarin Red clusters were smaller and stained less intensely in Type 2 pericytes as compared to Type 1 pericytes (Figure 4.4B). Finally, contrary to our hypothesis, we did not observe robust adipogenic differentiation in Type 1 pericytes. Instead, very few cells of Type 1 or Type 2 pericytes formed structures with a typical premature adipocyte morphology (intracellular lipid vesicles) and stained positive with a lipid dye (Figure 4.4C).

HFD Resulted in Weight Gain in Male but Not Female Mice

Final bodyweight for female mice was 50-60% lower than male mice (21.10 ± 1.00 , 21.03 ± 0.47 , 23.70 ± 0.74 , and 24.48 ± 1.01 vs. 34.75 ± 9.55 , 37.77 ± 1.05 , 42.65 ± 0.75 , and 38.70 ± 3.50 for females vs. males Con-Sed, Con-Ex, HFD-Sed and HFD-Ex, respectively, $p < 0.05$). Group by Sex two-way ANOVA yielded significant main effects of group and sex on weight gain (Figure 4.5A). Post hoc analysis revealed that compared to male mice, female mice gained significantly less weight (Sex main effect, $p < 0.05$). Moreover, mice in the HFD-Sed group gained more weight compared to Con-Sed and Con-Ex groups and mice in the HFD-Ex group also gained more weight than mice in the Con-Ex group (Group main effect, $p < 0.05$). No significant differences in body weight or weight gain were detected between the HFD-Sed and HFD-Ex groups.

The difference between the sexes in body weight and weight gain was also apparent in subcutaneous fat pad mass. Although no significant differences were found between the groups, female mice had significantly lower fat pad mass in all groups with the exception of HFD-Ex (Sex main effect, $p < 0.05$, Figure 4.5B).

No significant differences between the groups or the sexes were found in fasting glucose (Figure 4.5C), but both fasting insulin (Figure 4.5D) and HOMA (Figure 4.5E) were significantly different between males and females. Specifically, in both high fat groups (HFD-Sed and HFD-Ex) male mice had significantly higher fasting insulin and higher HOMA values compared to female mice ($p < 0.05$). No significant differences between the groups in fasting insulin or HOMA were detected.

Exercise Reversed the Increase in NG2⁺ Pericytes in High Fat Fed Male Mice

Since NG2⁺Nestin⁺ (Type 2) pericytes are extremely rare in adipose tissue (a few dozen events of 500,000 events) we analyzed NG2⁺ pericytes as a single population. We found that in male mice, HFD resulted in doubling of NG2⁺ pericytes (Group by Sex interaction, $p < 0.05$, Figure 4.6A). When purifying the population further by eliminating lineage cells, HFD tripled NG2⁺Lin⁻ pericytes in male mice (Group by Sex interaction, $p < 0.05$, Figure 4.6B). Exercise reversed the increase in NG2⁺ and NG2⁺Lin⁻ pericytes (Group by Sex interaction, $p < 0.05$, Figure 4.6). No changes in pericyte relative quantity were noted in female mice in response to HFD or exercise. A representing gating image is presented in Figure 4.6C.

No Changes in MSC Marker Expression in NG2⁺Lin⁻ Pericytes in Response to HFD or Exercise

The changes in NG2⁺Lin⁻ pericytes in response to high fat diet prompted us to investigate the expression of MSC markers in this population. The expression of CD90 and Sca-1 ranged from 70 to 90% of NG2⁺Lin⁻ pericytes (Figure 4.7A and C, respectively), whereas CD29 expression was lower and ranged from 25 to 40% of NG2⁺Lin⁻ pericytes (Figure 4.7B). No significant differences between the experimental groups were detected in the expression of CD90 and CD29 but Sca-1 expression in NG2⁺Lin⁻ pericytes was significantly lower in HFD-Ex

compared to all other groups (Figure 4.7C). No significant sex differences were detected as well in MSC marker expression. A representing gating image is presented in Figure 4.7D.

HFD Does Not Alter Adipocyte Precursor Marker Expression in NG2⁺ Pericytes

We next evaluated whether NG2⁺ pericytes increase their preadipocyte phenotype in response to HFD and whether under proper *in vivo* adipogenic stimulus (HFD) NG2⁺ pericytes could differentiate into adipocyte precursors. We utilized Berry and Rodeheffer [19] model and evaluated adipocyte precursors (Lin⁻CD29⁺CD34⁺Sca-1⁺). Gating hierarchy and a representing gating image are presented in Figure 4.8A and D, respectively. Adipose precursors (Lin⁻CD29⁺CD34⁺Sca-1⁺) ranged between 2 to 8% of NG2⁺ pericytes and this proportion was not significantly different across the different experimental groups (Figure 4.8B). The relative quantity of NG2⁺Lin⁻CD29⁺CD34⁺Sca-1⁺ pericytes within the SVF was extremely low. In female mice, NG2⁺Lin⁻CD29⁺CD34⁺Sca-1⁺ pericytes made up less than 0.1% of all cells and in male mice it made up under 0.3% of all stromal cells (Figure 4.8C). Similar to the increase in total NG2⁺Lin⁻ pericytes in male mice, NG2⁺Lin⁻CD29⁺CD34⁺Sca-1⁺ pericyte quantity increased in HFD-Sed males and was significantly different from Con-Ex. Exercise resulted in a decrease in NG2⁺Lin⁻CD29⁺CD34⁺Sca-1⁺ pericyte quantity in males and HFD-Ex pericyte quantity was significantly lower than Con-Sed and HF-Sed (Figure 4.8C).

PDGFR α Positive NG2⁺Lin⁻ Pericytes Are Extremely Rare in Adipose Tissue

Platelet Derived Growth Factor Receptor α positive (PDGFR α) cells ranged between 3 to 11% of NG2⁺Lin⁻ pericytes across the experimental groups and were not significantly different between the groups or sexes (Figure 4.9A). However, NG2⁺Lin⁻ PDGFR α ⁺ cells were extremely rare and made up less than 0.2% of all cells in all groups (Figure 4.9B). A representing gating image is presented in Figure 4.9C.

4.5 Discussion

The goal of this study was two-fold: 1) to identify and characterize Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes in adipose tissue and 2) to evaluate changes in surface marker expression in these two populations in response to HFD and to exercise. Type 1 and Type 2 pericytes were recently characterized in skeletal muscle, both types were associated with microvessels and expressed common pericyte markers (NG2, PDGFR β , and CD146) [8-10]. These cells were selected based on the expression of NG2, a common pericyte marker, and were separated into two distinct populations using Nestin (neural stem cell marker). Here, utilizing a similar methodology, we identified Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes in subcutaneous adipose tissue. We observed lower quantities of both Type 1 and Type 2 pericytes compared to previously reported [8-10]. Moreover, we found that contrary to previous reports in skeletal muscle [8-10], Type 1 pericytes were more abundant than Type 2 pericytes, and Type 2 pericytes were very rare in subcutaneous adipose tissue (less than 0.5% of isolated stromal cells). We also used flow cytometry to verify the expression of PDGFR β and CD146 in adipose-derived NG2⁺ pericytes and observed differential expression of PDGFR β between the two pericyte types. This difference was not reported previously, likely due to two methodological differences. 1) Birbrair *et al.* did not characterize adipose-derived pericytes and 2) the authors demonstrated the expression of PDGFR β and CD146 using histology and did not quantify the level of expression using flow cytometry or other means[8-10].

Our flow cytometry investigation revealed that in adipose tissue, as well as in skeletal muscle, the two NG2⁺ pericyte types express the endothelial marker CD31 (Figure 4.3E-F) and the hematopoietic marker CD45 (Figure 4.3G-H). CD31 expression was similar between skeletal muscle and adipose and ranged between ~15% in Type 1 to ~35% in Type 2 pericytes (Figure

4.3E). These data suggest that the co-localization images previously published [8,10] might in fact show co-expression of NG2 and CD31 in skeletal muscle and that the authors might have not isolated pure pericyte populations. Hence, when using NG2 as a marker for pericyte selection it is imperative to exclude hematopoietic (CD45⁺) and endothelial (CD31⁺) lineage cells (Lin) from the NG2⁺ fraction in order to isolate a pure population of pericytes (NG2⁺Lin⁻).

Birbrair and colleagues also evaluated differentiation capabilities of the two types of skeletal muscle-derived pericytes and reported that Type 1 pericytes are not myogenic but could undergo adipogenic differentiation while muscle-derived Type 2 pericytes are myogenic and not adipogenic [8]. Differentiation analysis in adipose-derived Type 1 and Type 2 pericytes reported here, suggests that adipose-derived Type 1 and Type 2 pericytes are not myogenic and that both are osteogenic. Unexpectedly, when analyzing adipogenic differentiation only very few cells in both Type 1 and Type 2 pericyte cultures stained positive with a lipid dye.

Pericytes are detected in most tissues [1] and can differentiate into several cell types, including osteocytes, chondrocytes, myocytes and adipocytes [3]. However, both pericyte morphology and phenotype could be different depending on the organ in which they reside as well as their developmental stage [20]. Using adipose-derived pericytes, we expected to detect robust adipogenic differentiation in response to adipogenic inducers in culture however adipogenic differentiation after 14 days in culture was not striking. Taken together, these data might suggest that NG2⁺ pericytes might have a different phenotype in adipose tissue compared to skeletal muscle and also due to the low quantity of Type 2 pericytes, that the Type 1-Type 2 dichotomy previously reported in skeletal muscle may not be applicable to adipose tissue

Based on findings from our initial investigation, mainly the extremely low quantity of NG2⁺Nestin⁺ (Type 2) pericytes in adipose tissue, we abandoned the Type1-Type2 classification

and isolated non-hematopoietic non-endothelial (Lin⁻) adipose-derived pericytes. We analyzed NG2⁺Lin⁻ pericytes and evaluated the effects of high fat diet on surface marker expression in this population. We also evaluated the efficacy of exercise in attenuating HFD-induced changes in NG2⁺Lin⁻ pericyte phenotype.

Moderately HFD (45% of calories from fat) for 18 weeks has been previously reported to result in weight gain and insulin resistance in male mice [13]. In this study we found that HFD resulted in significant weight gain but no changes in subcutaneous adipose mass or insulin resistance were detected. In male mice, compared to Con-Sed, fasting insulin and HOMA levels in HFD-Sed group were increased more than 4-fold but these differences were not statistically significant. Interestingly, female mice were not responsive to HFD and 18 wks of HFD did not result in weight gain, increased subcutaneous adipose mass or humoral measures of insulin resistance. Although in males, weight gain was lower in HFD-Ex compared to HFD-Sed (16.70±1.66 vs. 20.55±3.35, respectively) this difference was not statistically significant and unlike previously published work [13, 21-22], exercise did not significantly attenuate weight gain or insulin resistance in male or female mice.

Exercise might prevent adipocyte hypertrophy as demonstrated by Sakurai *et al.* [23]. These authors have reported that exercise training reduced *in vitro* adipogenesis from SVF cells extracted from rat epididymal fat pads. They also reported reduced lipid accumulation in SVF cells after adipogenic differentiation. However, in this study, young rats (14 weeks old at sacrifice) were used, and they were not subjected to a HFD [23]. In the current study, we report that HFD did not result in a significant increase in subcutaneous adipose mass and fat pad mass in adult mice.

In the current study, mice were subjected to forced treadmill exercise for 60 min a day, 5 days a week, this exercise volume is very low both in terms of the relative time mice were active (1 out of 24 hr in a day) and the total distance travelled (Less than 0.5 miles per session). Spontaneous housing-related activity that takes place in the mouse cage is increased in C57Bl/6 compared to other obesity-resistant strains [24]. Moreover, housing density affects housing related activity in rodents and individually housed rats were found to be less active compared to their group-housed controls [25]. Unlike other studies [13, 21-22] in which animals were individually-housed, animals in the current study were group-housed. These animals increased basal housing-related spontaneous activity could have resulted in similar total activity levels between the Ex and Sed groups. This might have confounded or blunted potential effects of physical activity on metabolic measures.

The impact of obesity on stem cell quantity and function has not been extensively explored. Two studies report no change in adipose stem cell content in response to obesity [14-15], while we previously found that HFD decreased Sca-1⁺CD45⁻ ADSC relative quantity in epididymal pads (Pincu *et al.* in progress, see Chapter 3 here). In the current study, although no significant changes were detected in measures of insulin resistance, the HFD induced increase in weight gain triggered a robust response in NG2⁺ pericyte quantity in male mice. A noticeable 3-fold increase in NG2⁺Lin⁻ pericyte quantity was detected in male HFD-Sed and was completely abolished in male HFD-Ex. In female mice, no changes were observed in response to HFD or exercise in NG2⁺ pericyte quantity. This significant sex difference in NG2⁺Lin⁻ pericyte response to HFD, exercise or their combination is likely impacted by the differences in weight gain. Female mice were not responsive to the diet and exercise intervention and it is likely that the

HFD induced stimulus that resulted in increased pericyte quantity in male adipose tissue was absent in female mice.

The increase in NG2⁺Lin⁻ pericyte quantity in response to HFD did not coincide with phenotypical changes, such as increase MSC- (Sca-1, CD90 and CD29) or adipogenic (PDGFR α) surface marker expression. We hypothesized that in response to HFD, a greater proportion of NG2⁺Lin⁻ pericytes will express MSC markers as they play role in maintaining adipose tissue plasticity [13-14], and HFD has been previously found to alter adipose stem cell function (i.e. multipotency and transcriptional activity) [15,26]. In this study, however, MSC marker expression (CD90 and CD29) in NG2⁺Lin⁻ pericytes did not differ between the experimental groups and was not different between female and male mice. Contrary to our hypothesis, the proportion of MSC within the pericyte population did not change in response to HFD or exercise, with the exception of Sca-1 which was significantly lower in HFD-Ex compared to all other groups. This suggests that the increased quantity of NG2⁺Lin⁻ pericytes in response to HFD results in a proportional increase in NG2⁺Lin⁻ MSCs without a shift in MSC relative quantity within the pericyte population.

Several studies support the concept that the source of adipose stem cells results in different function [27-29]. For example, adipose stem cells extracted from visceral depots (epididymal in mice or omental in humans) inherently demonstrate poor adipogenic differentiation potential and reduced proliferation compared to stem cells from subcutaneous depots [28-30]. Wu and colleagues [15], analyzed stem cells from different sources: bone marrow, inguinal and epididymal adipose and infrapatellar fat. The authors used different marker combination for the different cell sources. For inguinal and epididymal fat, Rodeheffer *et al.* [31] marker combination was used with some modification to eventually yield a Lin⁻Sca-1⁺CD34⁺

population [15]. Although finding no change in cell quantity, the authors report altered function in response to HFD [15].

Contrary to previously published work [27-28, 32], although not directly measuring pericyte function, in this study we report no change in pericyte phenotype in response to HFD. No increase in MSC marker expression in pericytes might suggest that 1) pericytes do not increase stromal activity (i.e trophic factor secretion) in response to HFD or 2) MSC marker positive pericyte respond to HFD by increasing secretion of factor without changing their proportion within the NG2⁺ pericyte population. The fact that NG2⁺ pericyte quantity was increased in response to HFD might support the latter.

We next addressed this question from another perspective and evaluated whether NG2⁺ pericytes increase their preadipocyte phenotype in response to HFD and whether under proper adipogenic stimulus (HFD) NG2⁺ pericytes could differentiate into adipocyte precursors. In an elegant Cre-recombinase study in which PDGFR α -Cre:mT/mG reporter mice were used to identify the adipocyte lineage, all adipocytes were labeled with the PDGFR α -Cre trace [19]. Based on their findings, Berry and Rodeheffer [19] proposed a model by which adipocyte progenitors (Lin⁻CD29⁺CD34⁺Sca-1⁺CD24⁺) lose the expression of CD24 and become preadipocytes (Lin⁻CD29⁺CD34⁺Sca-1⁺CD24⁻). We utilized this model and evaluated adipocyte precursors (Lin⁻CD29⁺CD34⁺Sca-1⁺) as an inclusive population which contains CD24⁺ adipocyte progenitors and CD24⁻ preadipocytes. Lin⁻CD29⁺CD34⁺Sca-1⁺ adipocyte precursors were 4-8% of NG2⁺ pericytes in males and 2-5% of NG2⁺ pericytes in females. Adipocyte precursor relative quantity within the pericyte population did not change in response to HFD, exercise or their combination in male or female mice. This concurs with our observation that MSC marker expression did not change as well in response to HFD or exercise.

It is important to note that NG2⁺ adipocyte precursors were extremely rare and comprised less than 0.3% of all stromal cells in subcutaneous adipose tissue. Moreover, the expression of PDGFR α , an essential adipogenic lineage marker [19], within NG2⁺Lin⁻ pericytes ranged between 3-6% in male and 5-10% in female mice but was less than 0.2% of all stromal cells in subcutaneous adipose tissue. Taken together with the lack of robust adipogenic differentiation *in vitro*, these data suggest that in subcutaneous adipose, NG2⁺ pericytes do not play a direct role in adipogenesis in response to HFD. We speculate that in response to an obesogenic stimulus in subcutaneous adipose tissue NG2⁺Lin⁻ pericytes proliferate and expand and not differentiate down the adipogenic lineage but play a role in angiogenesis and vascular remodeling by secreting growth factors and stimulating ECM remodeling [3,33]. This corroborates previous findings from our lab (Pincu *et al.*, in progress, see Chapter 3 here) that adipose-resident Sca-1⁺CD45⁻ (CD146⁺) stromal cells do not present an adipogenic gene expression profile in response to HFD but rather increase the expression of genes that control extracellular matrix remodeling.

Exercise training has been reported to decrease adipose tissue inflammation [11,13,22], to change macrophage polarization towards M2 phenotype [12], and to improve cellular oxidative homeostasis by increasing the resistance to oxidative stress [11]. Additionally, along with a controlled diet – exercise assists in weight loss and reducing adiposity [13]. Exercise promotes systemic changes that attenuate the metabolic co-morbidities of obesity [11,13,34]. Exercise training attenuates insulin resistance, reduces blood pressure and improves blood lipid profile [11]. Very few studies have addressed the interaction between adipose stem cells and the adipose microenvironment in the context of obesity and exercise training. However, the studies that do exist suggest that exercise might be beneficial to adipose stem cell function in obesity, through

modulation of adipose tissue hypoxia, fibrosis and attenuation of inflammation. In the current study, exercise did not attenuate weight gain, insulin resistance or pericyte phenotype (MSC and adipose precursor marker expression) but exercise did reverse the significant increase in NG2⁺Lin⁻ pericytes in response to HFD.

We speculate that the increased quantity of subcutaneous adipose-resident pericytes in response to HFD occurs in order to promote microenvironment changes to contain the persistent lipid influx as a result of the hypercaloric diet [7, 35-37]. Pericytes becomes activated, proliferate and secrete factors in order to promote angiogenesis and extracellular matrix remodeling in support of the expanding adipose tissue. Since exercise training hinders adipogenesis and adipocyte growth [23,38], the demand for adipose microenvironment changes decreases and so pericyte quantity returns to control levels.

4.6 Conclusion

This study provides the first investigation of Type 1 (NG2⁺Nestin⁻) and Type 2 (NG2⁺Nestin⁺) pericytes in subcutaneous adipose tissue and of subcutaneous adipose-resident NG2⁺ pericyte response to HFD, alone and in combination with exercise training in adult mice. Contrary to previous reports in skeletal muscle [8-10], Type 2 (NG2⁺Nestin⁺) pericytes were very rare in subcutaneous adipose tissue and Type 1 (NG2⁺Nestin⁻) pericyte quantity was higher than Type 2 pericytes. Consequently, we abandoned the Type1-Type2 classification and isolated non-hematopoietic non-endothelial (Lin⁻) adipose-derived NG2⁺ pericytes.

Contrary to our hypothesis, NG2⁺Lin⁻ pericyte quantity in males was increased in response to HFD and was attenuated in response to exercise. This increase however, did not coincide with phenotypical changes, (such as altered MSC marker expression or increased adipogenic lineage marker expression). Taken together with a lack of robust adipogenic

differentiation *in vitro*, we conclude that in subcutaneous adipose, NG2⁺ pericytes do not play a direct role in adipogenesis in response to HFD. We speculate that in response to an obesogenic stimulus NG2⁺Lin⁻ pericytes in subcutaneous adipose tissue do not contribute to adipogenesis, but support ECM remodeling and angiogenesis necessary for tissue expansion. This corroborates previous findings from our lab (Pincu *et al*, in progress, see Chapter 3 here) that adipose-resident Sca-1⁺CD45⁻ (CD146⁺) stromal cells do not present an adipogenic gene expression profile in response to HFD but rather increase the expression of genes that control extracellular matrix remodeling.

4.7 References

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4.8 Figure Captions and Figures

Figure 4.1

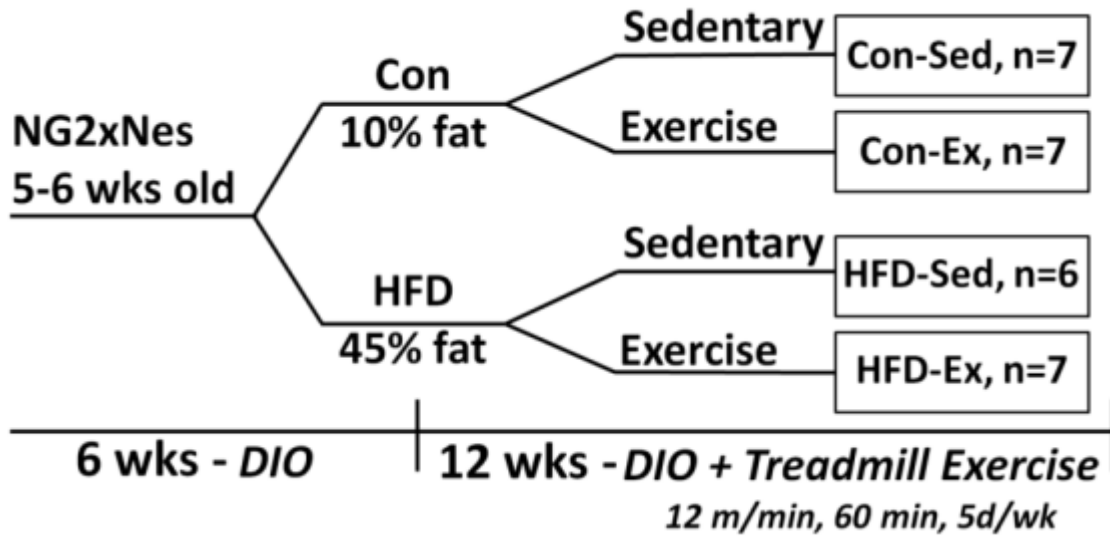


Figure 4.1 Study design. Mice were randomly assigned to one of four groups and were fed CON or HFD diets for 6 weeks. Mice then remained sedentary or were subjected to a 12 week treadmill exercise program while maintaining their diets. Con, Control diet; HFD, High fat diet; Sed, Sedentary (no exercise); Ex, Exercise; DIO, Diet-induced obesity.

Figure 4.2

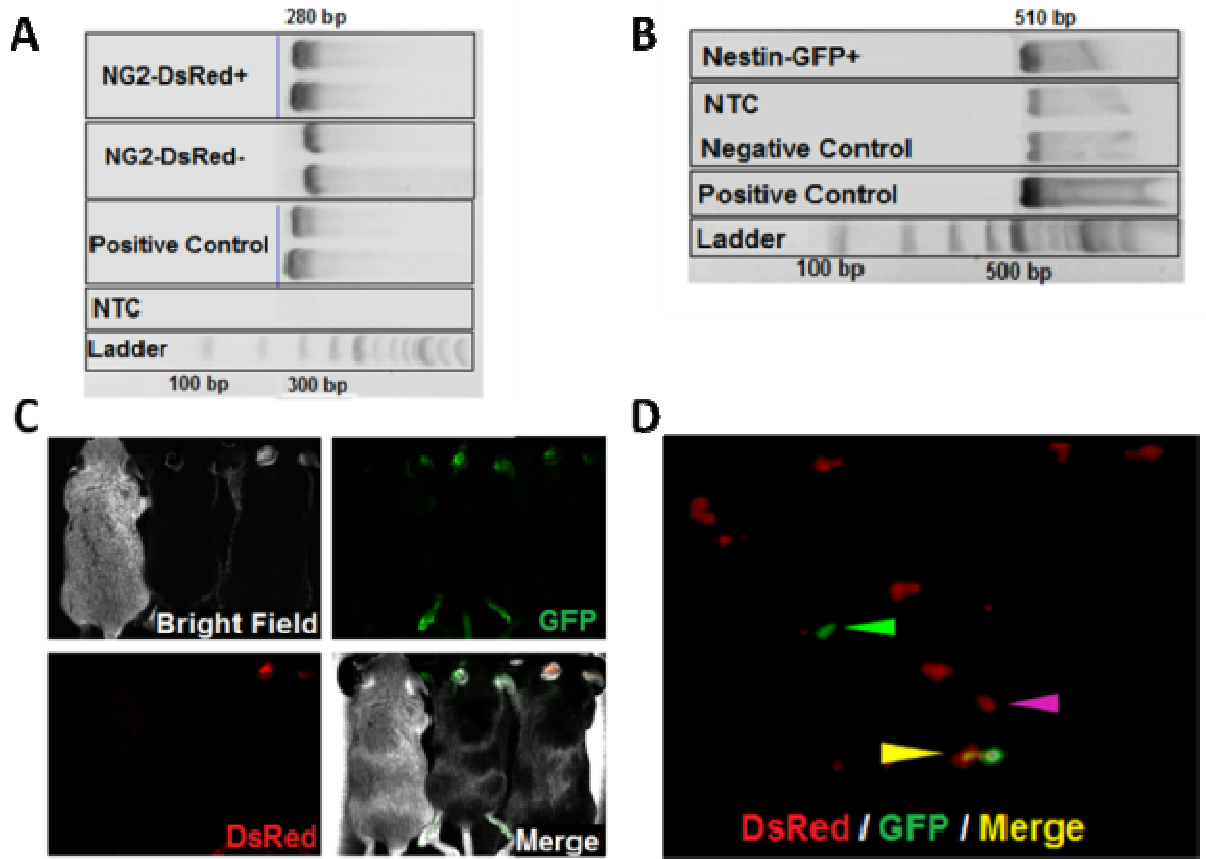


Figure 4.2 Verification of Fluorescent Protein Expression. Mice were genotyped for NG2-DsRed (A) and Nestin-GFP (B). Fluorescent protein expression was verified using *in vivo* macro imaging system (C). Adipose stromal cells were extracted from NG2xNes mouse, cultured overnight and inspected under a fluorescent microscope (10X) for detection of NG2-DsRed⁺Nestin-GFP⁻ (Purple arrowhead) or NG2-DsRed⁺Nestin-GFP⁺ cells (Yellow arrowhead). NG2-DsRed⁻ Nestin-GFP⁺ cells are also indicated (Green arrowhead) (D).

Figure 4.3

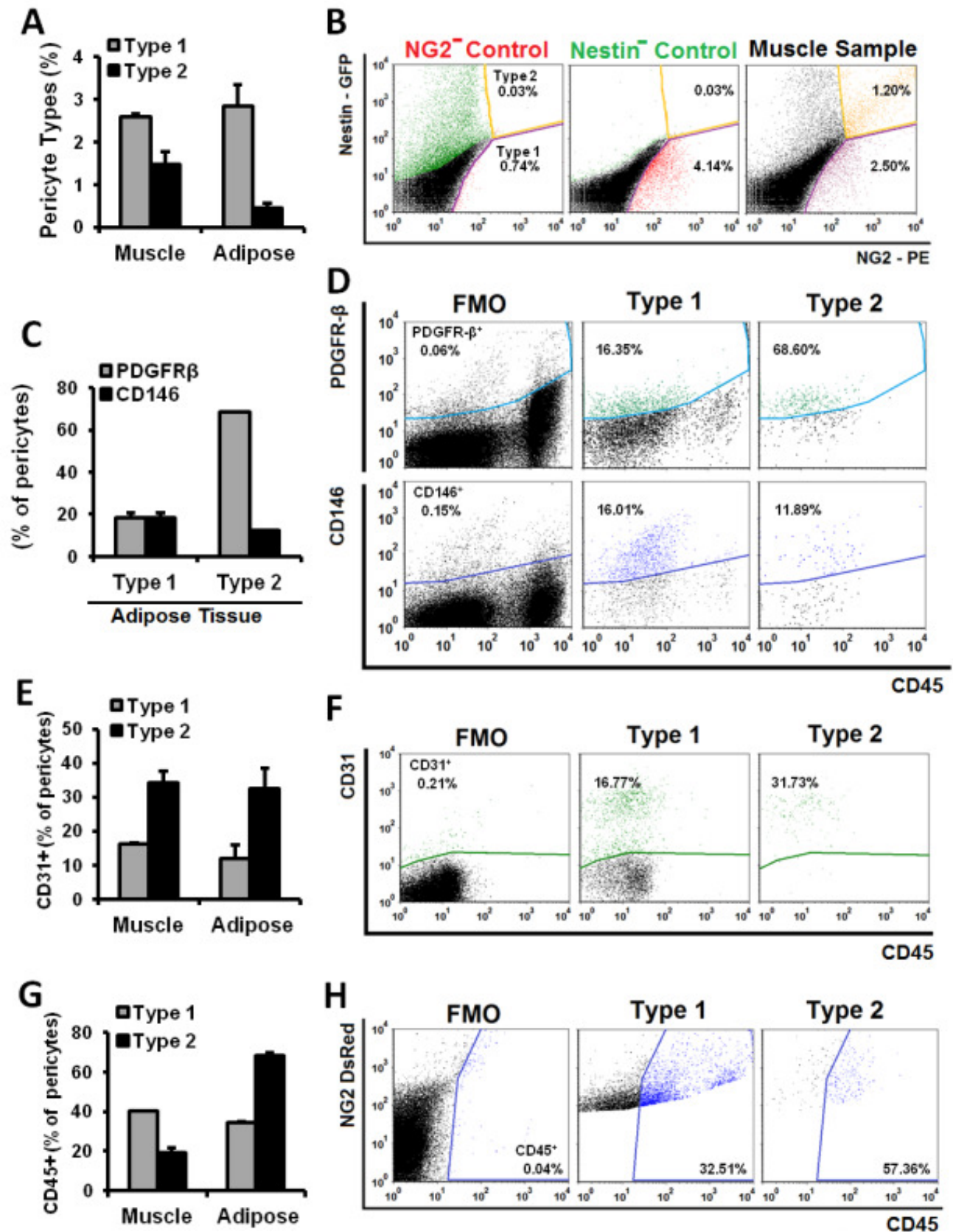


Figure 4.3 Flow Cytometry Analysis of Pericyte Types in Skeletal Muscle and Adipose Tissue. Quantification of NG2⁺Nestin⁻ (Type 1) or NG2⁺Nestin⁺ (Type 2) pericytes in skeletal muscle and subcutaneous adipose (A) and a representative image of flow analysis (B). Relative expression of PDGFR β and CD146 in subcutaneous adipose-derived Type 1 and Type 2 pericytes (C) and a representative image of flow analysis (D). Relative expression of CD31 (E) and CD45 (G) in skeletal muscle- and subcutaneous adipose-derived Type 1 and Type 2 pericytes (C) and representative images of flow analysis of CD31 (F) and CD45 (H). n=2 mice. All values are mean \pm SEM. FMO, Fluorescence minus one (negative control for a respective fluorescent marker within a multi marker panel).

Figure 4.4

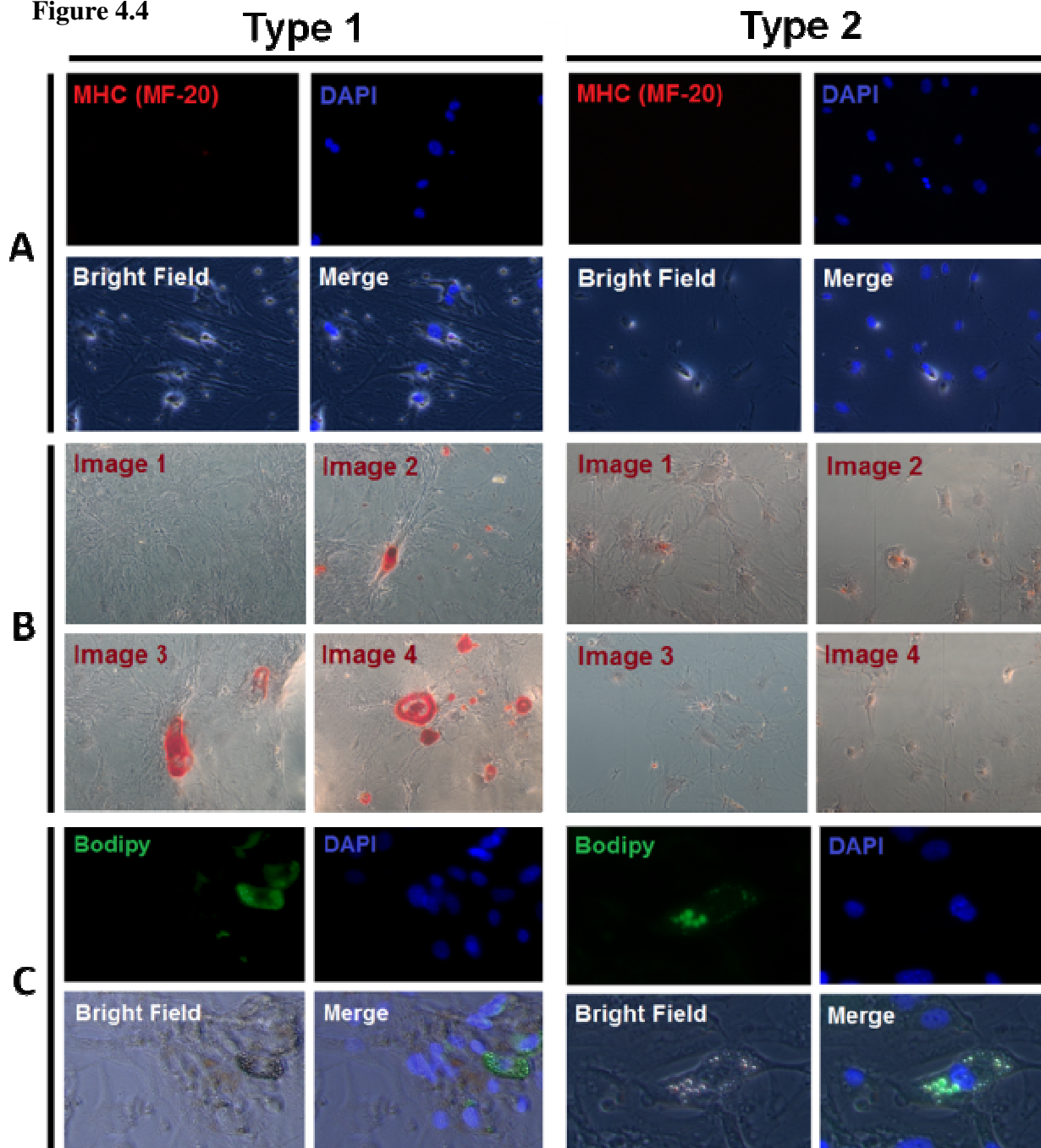


Figure 4.4 Tri-Lineage Differentiation Analysis in Adipose-Derived Type 1 and Type 2 Pericytes. Representative images of differentiation analysis in adipose-derived Type 1 (left panel) and Type 2 (right panel) pericytes. Expression of Myosin heavy chain (MF-20, Red) counterstained nuclei (DAPI, blue), cellular orientation (Bright field) and merged images at 20X magnification are illustrated (A). Four different Alizarin Red stain images per pericyte type are presented (B). Presence of lipids (Bodipy, Green) counterstained nuclei (DAPI, blue), cellular orientation (Bright field) and merged images at 20X magnification are illustrated (C).

Figure 4.5

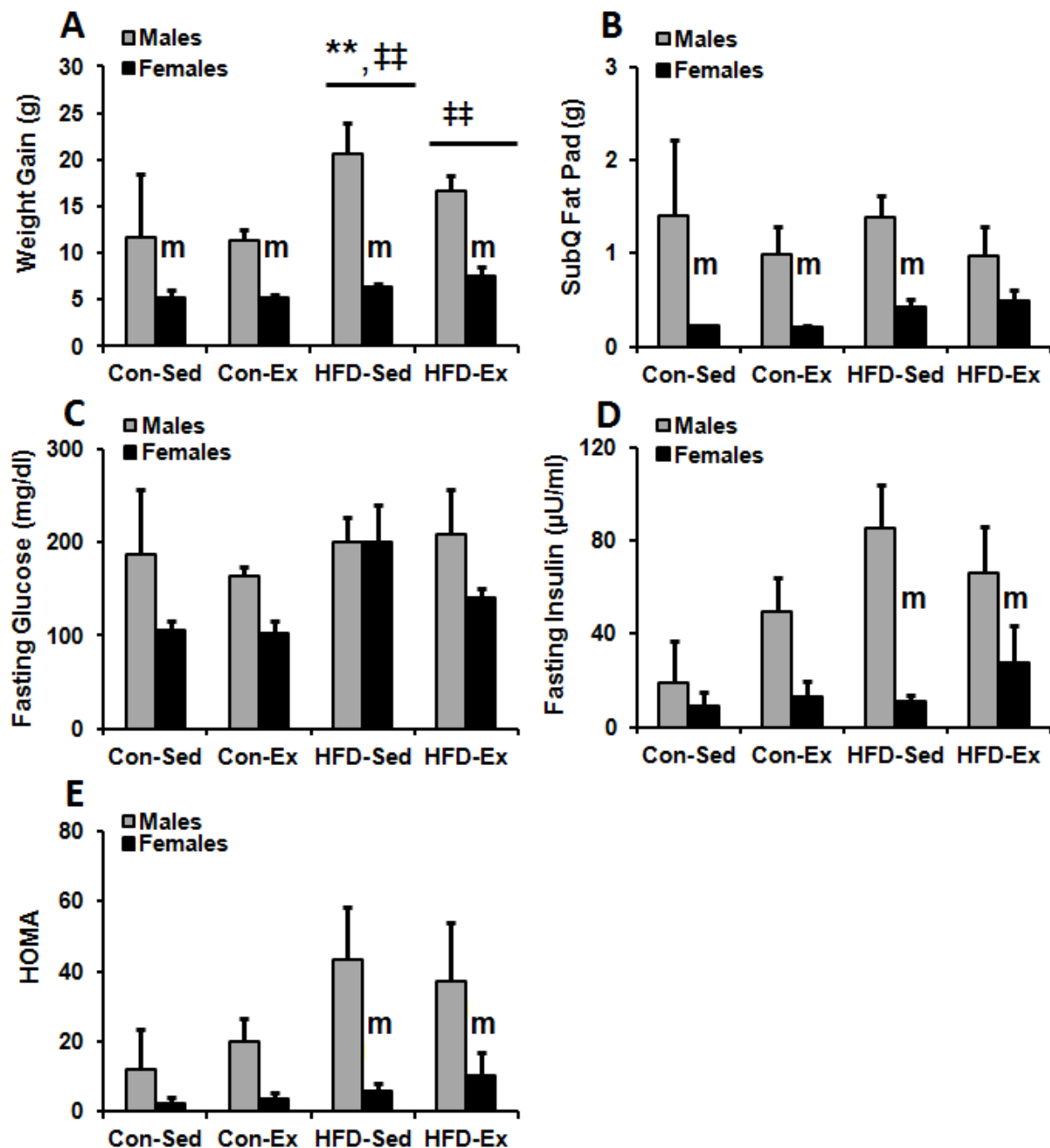


Figure 4.5 HFD Resulted in Weight Gain in Male but Not Female Mice. Weight gain (A) and subcutaneous adipose mass (B) were measured in grams. Fasting plasma glucose (C) was measured using a colorimetric assay. Fasting plasma insulin (D) was measured using an ELISA kit. Homeostasis model assessment of insulin resistance (HOMA) (E) was calculated from fasting insulin and glucose values. Con-Sed, n=4 females/3 males, Con-Ex, n=4 females/3 males, HFD-Sed, n=3 females/3 males and HFD-Ex, n=4 females/3 males. All values are mean \pm SEM. Group main effects are annotated, ** = $p < 0.05$ vs. Con-Sed, $\ddagger\ddagger$ = $p < 0.05$ vs. Con-Ex. Sex main effect is annotated, **m**= $p < 0.05$ vs. Males in the same Treatment Group.

Figure 4.6

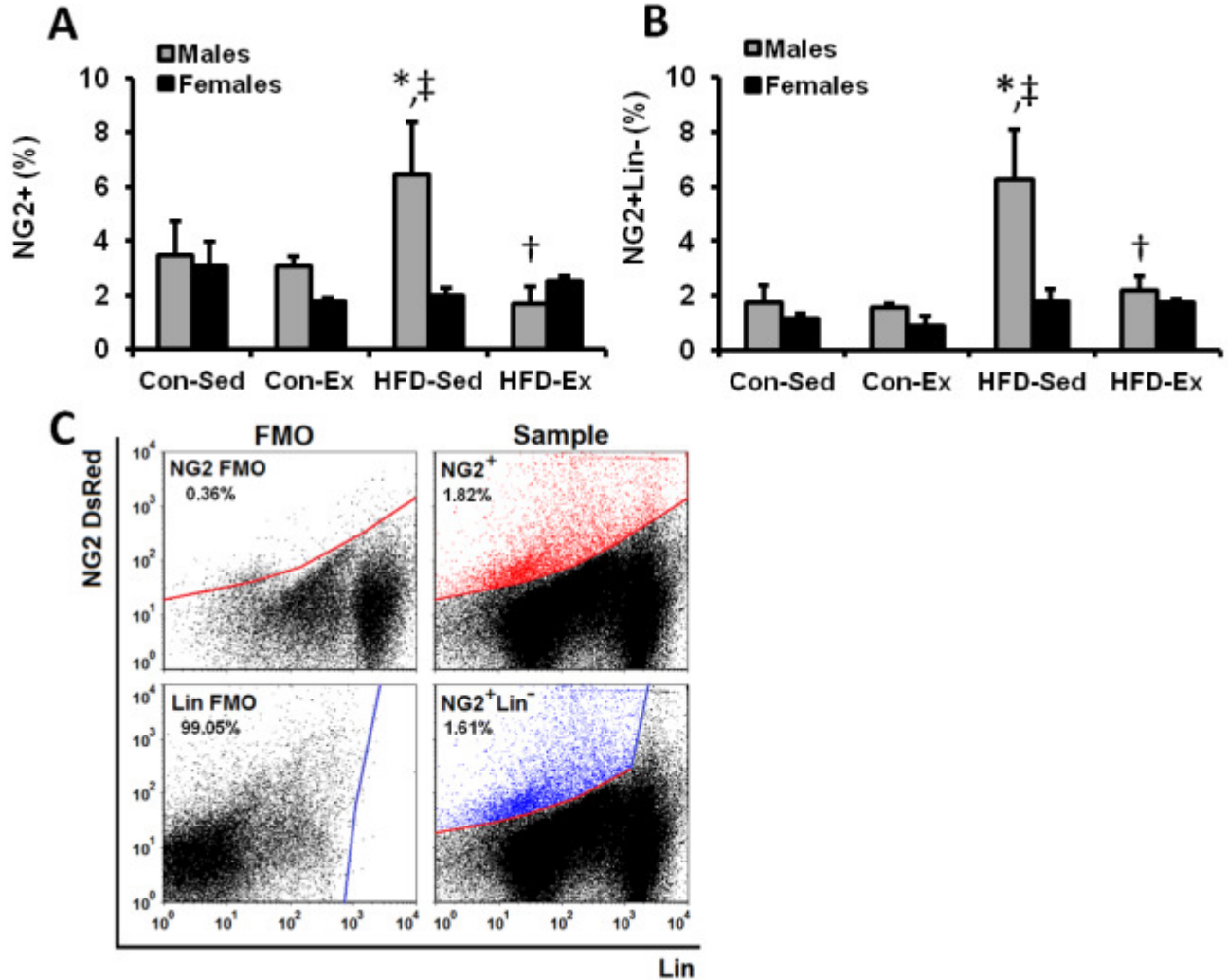


Figure 4.6 Exercise Reversed the Increase in NG2⁺ Pericytes in High Fat Fed Male Mice. Relative quantity of subcutaneous adipose-derived NG2⁺ (A) or NG2⁺Lin⁻ (B) pericytes in males and females across the four experimental groups and a representative image of flow analysis (C). Con-Sed, n=4 females/3 males, Con-Ex, n=4 females/3 males, HFD-Sed, n=3 females/3 males and HFD-Ex, n=4 females/3 males. All values are mean \pm SEM. Sex by Group interaction is annotated, * = $p < 0.05$ vs. Con-Sed in the same Sex, ‡ = $p < 0.05$ vs. Con-Ex in the same Sex, † = $p < 0.05$ vs. HF-Sed in the same Sex. FMO, Fluorescence minus one (negative control for a respective fluorescent marker within a multi marker panel).

Figure 4.7

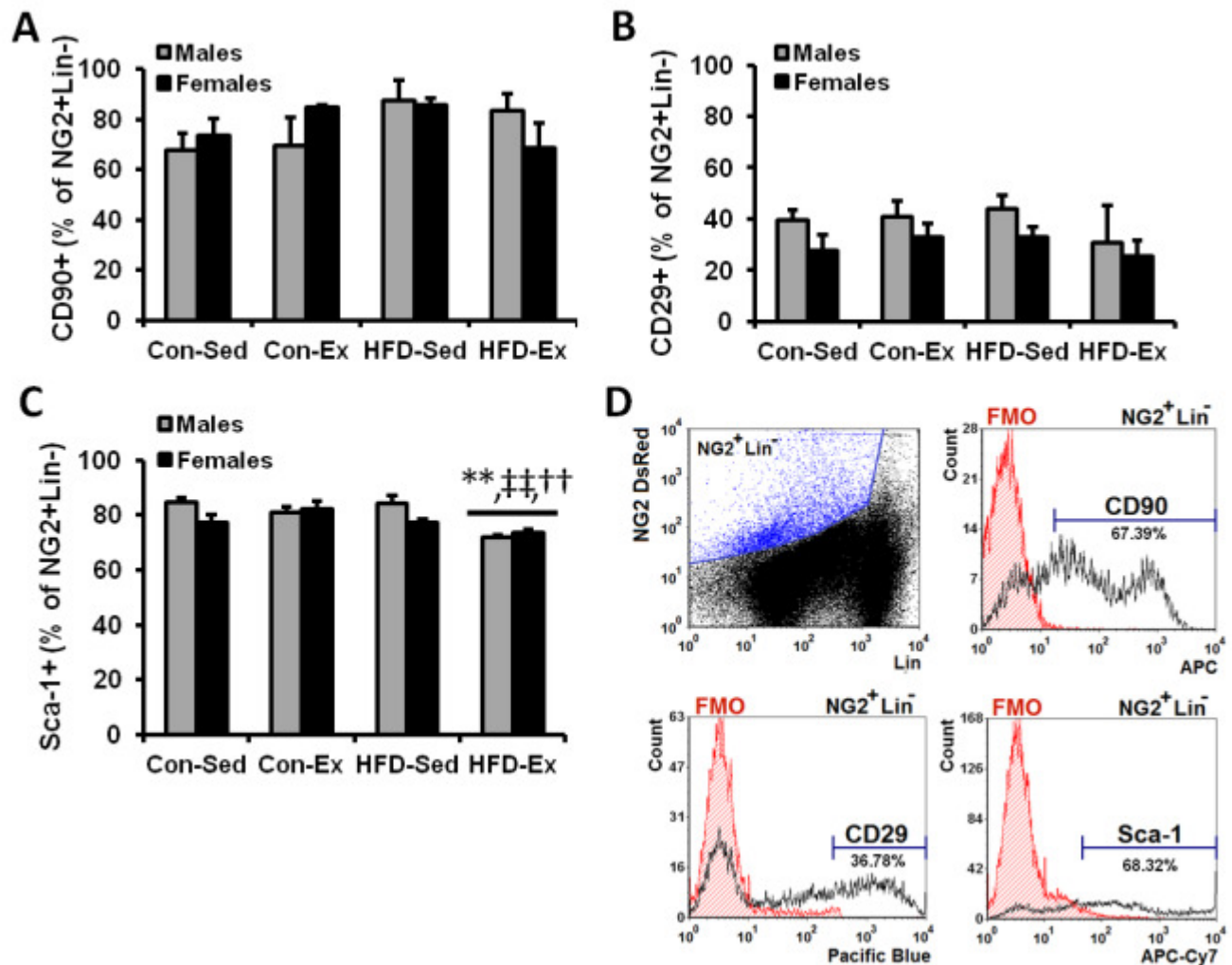


Figure 4.7 No Changes in MSC Marker Expression in NG2⁺Lin⁻ Pericytes in Response to HFD or Exercise. Relative expression of CD90 (A), CD29 (B) and Sca-1 (C) in subcutaneous adipose-derived NG2⁺Lin⁻ pericytes in males and females across the four experimental groups and a representative image of flow analysis (D). Con-Sed, n=4 females/3 males, Con-Ex, n=4 females/3 males, HFD-Sed, n=3 females/3 males and HFD-Ex, n=4 females/3 males. All values are mean \pm SEM. Group main effects are annotated, ** = p<0.05 vs. Con-Sed, ‡‡= p<0.05 vs. Con-Ex, ††= p<0.05 vs. HF-Sed. FMO, Fluorescence minus one (negative control for a respective fluorescent marker within a multi marker panel).

Figure 4.8

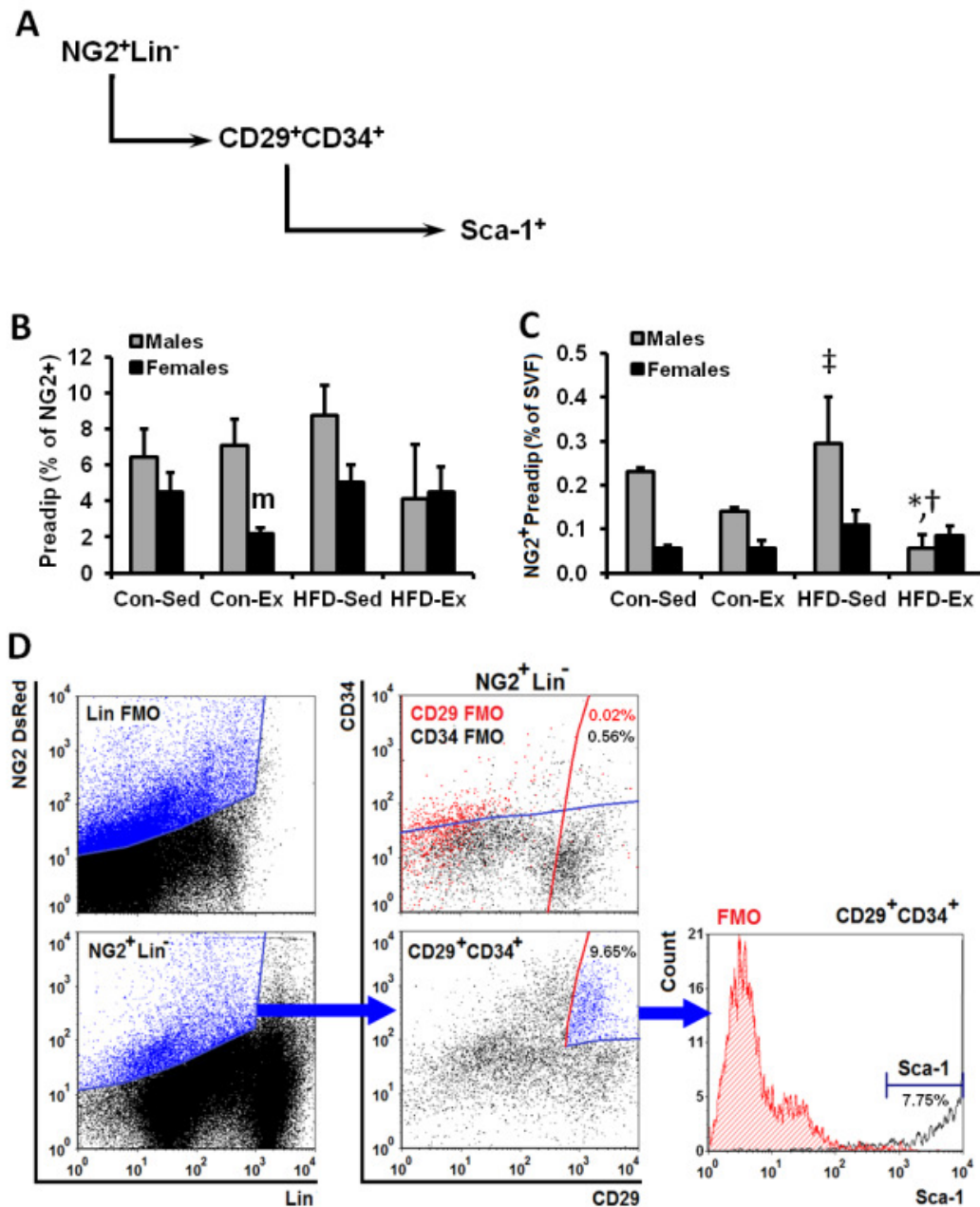


Figure 4.8 HFD Does Not Alter Adipocyte Precursor Marker Expression in NG2⁺ Pericytes. Gating hierarchy is presented for adipose precursors (A). Relative quantity of adipose precursors within the NG2⁺ pericytes (B), relative quantity of NG2⁺Lin⁻CD29⁺CD34⁺Sca-1⁺ cells within the subcutaneous adipose SVF (C) in males and females across the four experimental groups and a representative image of flow analysis (D). Con-Sed, n=4 females/3 males, Con-Ex, n=4 females/3 males, HFD-Sed, n=3 females/3 males and HFD-Ex, n=4 females/3 males. All values are mean \pm SEM. Sex by Group interaction is annotated, * = p<0.05 vs. Con-Sed in the same Sex, ‡ = p<0.05 vs. Con-Ex in the same Sex, † = p<0.05 vs. HF-Sed in the same Sex. Sex main effect is annotated, m=p<0.05 vs. Males in the same Treatment Group. FMO, Fluorescence minus one (negative control for a respective fluorescent marker within a multi marker panel).

Figure 4.9

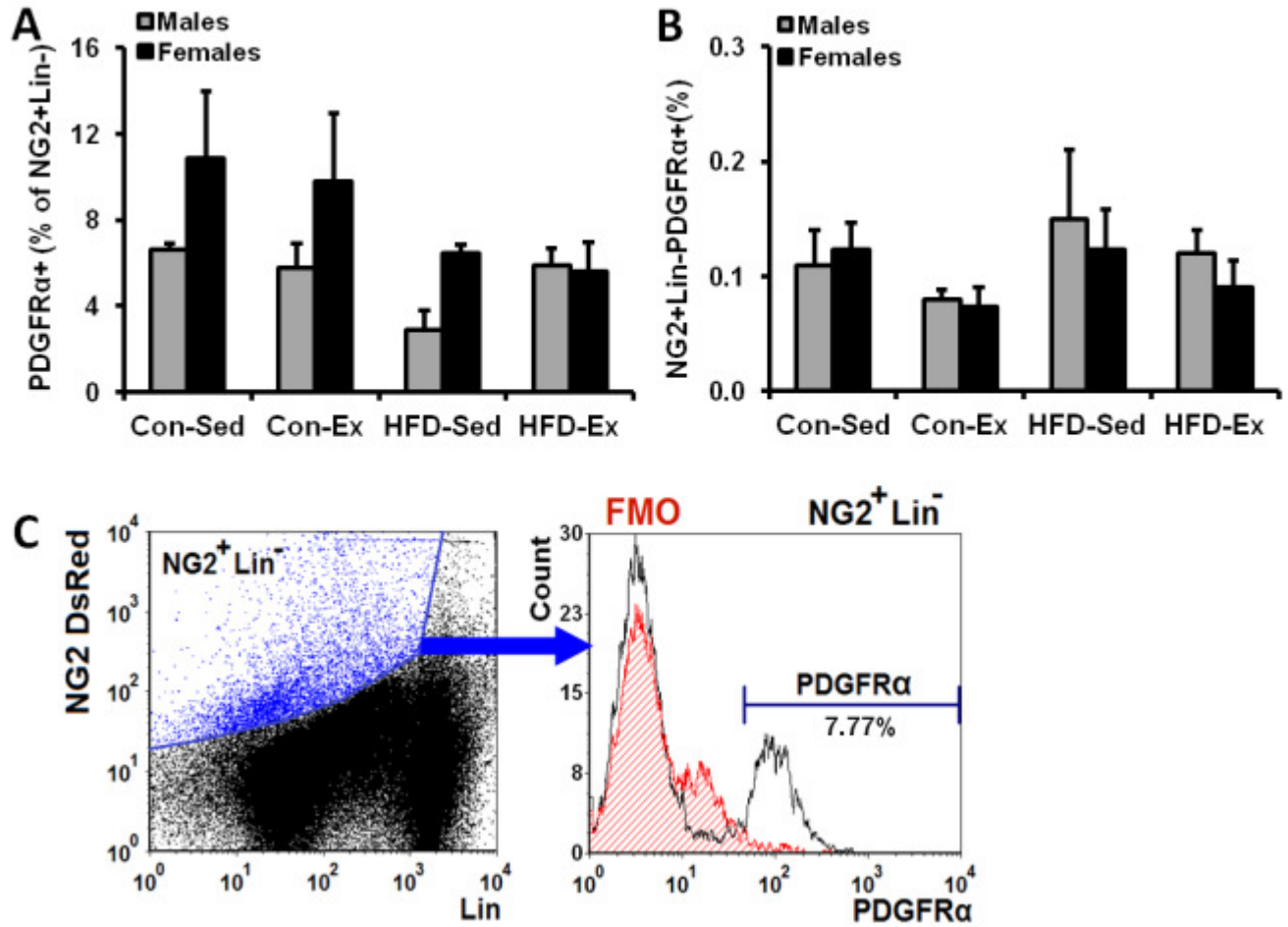


Figure 4.9 PDGFR α Positive NG2⁺Lin⁻ Pericytes Are Extremely Rare in Adipose Tissue. Relative expression of PDGFR α within the subcutaneous adipose-derived NG2⁺Lin⁻ pericyte population (A), relative quantity of NG2⁺Lin⁻PDGFR α ⁺ pericytes within the subcutaneous adipose stromal cell population (B) in males and females across the four experimental groups and a representative image of flow analysis (C). Con-Sed, n=4 females/3 males, Con-Ex, n=4 females/3 males, HFD-Sed, n=3 females/3 males and HFD-Ex, n=4 females/3 males. All values are mean \pm SEM. FMO, Fluorescence minus one (negative control for a respective fluorescent marker within a multi marker panel).

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of these studies was to examine the impact of obesity on adipose-resident stem/stromal cell quantity and function, and evaluate the extent to which exercise can attenuate these changes. We demonstrated that 60% fat diet results in insulin resistance, epididymal adipose inflammation and fibrosis and that these changes coincide with a decrease in epididymal ADSC (MSC) quantity and increased expression of genes related to ECM remodeling. A 45% fat diet increased the quantity of subcutaneous adipose-derived NG2⁺Lin⁻ pericytes in male but not female mice. No phenotypical changes were detected in either sex. Exercise restored NG2⁺Lin⁻ pericyte quantity in subcutaneous adipose, but did not change Sca-1⁺CD45⁻ stromal cell quantity and also did not have striking effects on function in either population.

Although we did not perform a direct and controlled comparison between the two studies, our findings suggest that adipose-resident stem/stromal cell response to obesity depends on the source of the cells (skeletal muscle, epididymal or inguinal adipose tissue) and the surface marker combination used to obtain the cells (Sca-1⁺CD45⁻ vs. NG2⁺Lin⁻). Additionally, sex might also play an important role in the adipose-resident stem/stromal cell response to obesity.

Importantly, we detected Type 1 and Type 2 pericytes in subcutaneous adipose. However, since Type 2 pericytes were extremely rare in adipose tissue and both pericyte types had significant endothelial (CD31) and hematopoietic (CD45) marker expression, we abandoned the Type 1- Type 2 classification and instead analyzed NG2⁺Lin⁻ pericytes. Our findings emphasize the importance of using a negative selection criterion for lineage markers when obtaining these cells.

In conclusion, the results of these studies suggest that adipose-derived stem/stromal cells from different adipose depots are differently impacted by high fat diet and exercise. Moreover, subcutaneous adipose tissue is a preferred site for stem cell harvesting since adipose tissue is extremely plastic and abundant and it is likely to be less negatively affected by obesity.

Further studies are necessary to investigate the following questions:

- 1) What is the mechanism that results in NG2⁺Lin⁻ pericyte quantity increase in response to high fat diet-induced obesity?
- 2) What is the mechanism that results in NG2⁺Lin⁻ pericyte quantity decrease in response to exercise in obese mice?
- 3) What is the extent to which high fat diet induced obesity impact NG2⁺Lin⁻ pericyte quantity in visceral adipose tissue?
- 4) What is the extent to which sex impacts adipose-derived stem cell phenotype and response to obesity and/or exercise?
- 5) What is the extent to which obesity-related changes alter the ability of adipose-derived NG2⁺Lin⁻ pericytes to remodel injured tissue after being injected into the damaged tissue?